

Class I. **Alphaproteobacteria** class. nov.

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Al.pha.pro.te.o.bac.te'ri.a. Gr. n. *alpha* name of first letter of Greek alphabet; Gr. n. *Proteus* ocean god able to change shape; Gr. n. *bakterion* a small rod; M.L. fem. pl. n. *Alphaproteobacteria* class of bacteria having 16S rRNA gene sequences related to those of the members of the order *Caulobacterales*.

The class *Alphaproteobacteria* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the class contains the orders *Caulobacterales*, “*Parvularculales*”, *Rhizo-*

biales, *Rhodobacterales*, *Rhodospirillales*, *Rickettsiales*, and *Sphingomonadales*.

Type order: **Caulobacterales** Henrici and Johnson 1935a, 4.

Order I. **Rhodospirillales** Pfennig and Truper 1971, 17^{AL}

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Rho.do.spi.ril.la'les. M.L. neut. n. *Rhodospirillum* type genus of the order; *-ales* suffix to denote order; M.L. fem. n. *Rhodospirillales* the *Rhodospirillum* order.

The order *Rhodospirillales* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the order contains the families *Rhodospirillaceae* and *Acetobacteraceae*.

Order is morphologically, metabolically, and ecologically diverse. Includes chemoorganotrophs, chemolithotrophs, and fac-

ultative photoheterotrophs; some of the latter are also able to grow photoautotrophically. Other species can grow methylo-

Type genus: **Rhodospirillum** Molisch 1907, 24 emend. Imhoff, Petri and Süling 1998, 796.

Family I. **Rhodospirillaceae** Pfennig and Trüper 1971, 17^{AL}

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Rho.do.spi.ril.la'ce.ae. M.L. neut. n. *Rhodospirillum* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Rhodospirillaceae* the *Rhodospirillum* family.

The family *Rhodospirillaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Rhodospirillum* (type genus), *Azospirillum*, *Inquilinus*, *Levispirillum*, *Magnetospirillum*, *Phaeospirillum*, *Rhodocista*, *Rhodospira*, *Rhodovibrio*, *Roseospira*, *Skermanella*, *Thalassospira*, and *Tistrella* (type genus). *Inquilinus*, *Thalassospira*, and *Tistrella* were proposed after the cut-off date for inclusion in this volume (June 30, 2001) and are not described here (see Coenye et al., 2002; López-López et al., 2002; and Shi et al., 2002, respectively).

Preferred mode of growth for most genera is photoheterotrophic under anoxic conditions in light. Grow chemotrophically in the dark. *Azospirillum*, *Magnetospirillum*, and *Skermanella* are chemoorganotrophic. Motile by means of polar flagella; may have lateral flagella.

Type genus: **Rhodospirillum** Molisch 1907, 24 emend. Imhoff, Petri and Süling 1998, 796.

Genus I. Rhodospirillum Molisch 1907, 24^{AL} emend. Imhoff, Petri and Süling 1998, 796

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Rho.do.spi.ril'lum. Gr. n. *rhodon* the rose; M.L. neut. n. *Spirillum* a bacterial genus; M.L. neut. n. *Rhodospirillum* the rose *Spirillum*.

Cells are vibrioid to spiral shaped, are motile by means of bipolar flagella, and multiply by binary fission. **Gram negative, belonging to the Alphaproteobacteria.** Internal photosynthetic membranes are present as vesicles or as lamellae forming a sharp angle to the cytoplasmic membrane. Photosynthetic pigments are **bacteriochlorophyll a** (esterified with phytol or geranylgeraniol) and **carotenoids of the spirilloxanthin series** with spirilloxanthin itself lacking in some species. **Ubiquinones and rhodoquinones with 8 or 10 isoprene units** are present. **Major cellular fatty acids are**

C_{18:1}, C_{16:1}, and C_{16:0}, with C_{18:1} as dominant component (51–55% of total fatty acids).

Grow preferentially photoheterotrophically under anoxic conditions in the light. **Photoautotrophic growth with molecular hydrogen and sulfide** as photosynthetic electron donors may occur. **Chemotrophic growth occurs under microoxic to oxic conditions in the dark.** Some species are very sensitive to oxygen; others grow equally well aerobically in the dark. **Fermentation and oxidant-dependent growth may occur.** Polysaccharides, poly-β-hy-

droxybutyric acid and polyphosphates may be present as storage products. Growth factors required. **Mesophilic freshwater bacteria with preference for neutral pH.**

The mol% G + C of the DNA is: 62.1–63.5.

Type species: ***Rhodospirillum rubrum*** (Esmarch 1887) Molisch 1907, 25 (*Spirillum rubrum* Esmarch 1887, 230.)

FURTHER DESCRIPTIVE INFORMATION

Two species of *Rhodospirillum* are currently known. *Rhodospirillum rubrum*, the type species, is one of the most intensively studied of the phototrophic bacteria. Numerous investigations on the physiology and enzymology of this species have focused on its metabolic properties, in particular CO₂ fixation and characterization of ribulose-1,5-bisphosphate carboxylase (Tabita, 1995), ATP generation and coupling-factor ATPase (Gromet-Elhanan, 1995), and nitrogenase and nitrogen fixation (Ludden and Roberts, 1995).

R. rubrum grows very well under photoheterotrophic conditions, but it can also grow under photoautotrophic conditions with molecular hydrogen (Klemme, 1968) or sulfide as the electron donor if supplied at low concentrations (Hansen and van Gernerden, 1972). Autotrophic CO₂ fixation is well documented and occurs via ribulose-1,5-bisphosphate carboxylase (Anderson and Fuller, 1967a, b). This enzyme has been highly purified and is well characterized (e.g., Tabita and McFadden, 1974a, b; Tabita, 1995). Ribulose-1,5-bisphosphate carboxylase is derepressed only at low CO₂ tensions (1.5–2.0% of the atmospheric tension) and can make up to 50% of the total soluble protein of the cells under such conditions (Sarles and Tabita, 1983). In the presence of malate or acetate, however, CO₂ is not assimilated via the reductive pentose phosphate cycle, but by other carboxylating reactions.

Under anoxic dark conditions, *R. rubrum* is able to ferment sugars and pyruvate (Kohlmiller and Gest, 1951; Gürgün et al., 1976; Gorrell and Uffen, 1977). Pyruvate is cleaved by pyruvate formate lyase, which is specifically induced under these growth conditions (Uffen, 1973; Jungermann and Schön, 1974). From formate, H₂ and CO₂ are formed by a CO-sensitive formic hydrogen lyase (Gorrell and Uffen, 1977). H₂, CO₂, acetate, and eventually propionate are produced as fermentation products from pyruvate. *R. rubrum* is able to gain energy from the coupling of CO oxidation and H₂ evolution and induces the synthesis of a carbon monoxide dehydrogenase on exposure to CO under anoxic conditions (Bonam et al., 1989; Kerby et al., 1995).

Rhodospirillum rubrum is also able to perform an anaerobic dark metabolism with DMSO and trimethylamine-N-oxide as electron acceptors (Schultz and Weaver, 1982). Under these conditions, growth is possible with succinate, malate, and acetate as substrates, and CO₂ and DMSO or trimethylamine are formed (Schultz and Weaver, 1982).

Rhodospirillum species can grow with ammonia or N₂ as sole nitrogen source (Siefert, 1976; Madigan et al., 1984). The nitrogenase of *R. rubrum* is subject to post-translational inactivation by ADP-ribosylation under energy-limiting conditions and if fixed nitrogen compounds are available (see Ludden and Roberts, 1995). Ammonia assimilation is mediated by the glutamine synthetase/glutamate synthase reactions, which are NADPH-dependent in *R. rubrum* (Brown and Herbert, 1977). Although a nitrate reductase is present in *R. rubrum*, growth with nitrate as sole nitrogen source apparently is not possible (Katoh, 1963; Taniguchi and Kamen, 1963; Ketchum and Sevilla, 1973;

Klemme, 1979). Purines can be used as a nitrogen source under anoxic conditions in the light and under oxic conditions in the dark (Aretz et al., 1978).

Comparably few studies have been performed with *Rhodospirillum photometricum* (Pfennig et al., 1965; Lehmann, 1976; Sarkar and Banerjee, 1980), which is very sensitive to oxygen and does not grow under oxic conditions in the dark (as does *R. rubrum*; Pfennig, 1969b), but only under microoxic conditions, provided the oxygen tension is lower than 0.5 kPa (Lehmann, 1976). Like *Phaeospirillum* species, *R. photometricum* is presumably unable to induce a second electron transport chain in the presence of oxygen and therefore depends on microoxic conditions in which the internal membrane system and the light-driven electron transport chain are fully expressed. Accordingly, the cells are fully pigmented under these growth conditions (Lehmann, 1976).

Two different types of alcohols are esterified with the bacteriochlorophyll *a* of *Rhodospirillum* species. Besides phytol, which is present as an alcohol in the majority of the Purple Nonsulfur Bacteria (PNSB), geranylgeraniol is the major component of *R. rubrum* and of some strains of *R. photometricum* (Brockmann and Knobloch, 1972; Künzler and Pfennig, 1973). Carotenoids of the spirilloxanthin series are present in *Rhodospirillum* species, but *R. photometricum* is unable to synthesize the end product, spirilloxanthin, and accumulates intermediates of this pathway, such as lycopene, rhodopin, anhydrorhodovibrin, and rhodovibrin (Schmidt, 1978).

Growth of *R. photometricum* is completely inhibited at penicillin concentrations of only 10 U/ml, whereas in *R. rubrum*, complete inhibition occurs at penicillin concentrations of more than 1000 U/ml (Weaver et al., 1975a).

ENRICHMENT AND ISOLATION PROCEDURES

Media and growth conditions used for isolation and cultivation of freshwater PNSB in general can also be applied for *Rhodospirillum* species. Various recipes for appropriate media have been developed in different laboratories (see Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Trüper, 1992). One of these, a mineral salts medium that has been used for cultivation of the great majority of PNSB over many years is given in the footnote below.¹ Standard techniques for the isolation of anaerobic bacteria in agar dilution series and on agar plates can be applied for *Rhodospirillum* species (Biebl and Pfennig, 1981; Imhoff and Trüper, 1992), if care is taken to establish and maintain oxygen-free conditions, especially for oxygen-sensitive species. This can be achieved by addition of 0.05% sodium ascorbate or 0.025% thioglycolate to the growth media in completely filled screw-capped bottles.

1. AT medium contains (g/l): KH₂PO₄, 1.0; MgCl₂·6H₂O, 0.5; CaCl₂·2H₂O, 0.1; NH₄Cl, 1.0; NaHCO₃, 3.0; Na₂SO₄, 0.7; NaCl, 1.0; sulfate-free trace element solution SLA (Imhoff and Trüper, 1977; Imhoff, 1992), 1 ml; and vitamin solution VA (Imhoff and Trüper, 1977; 1992), 1 ml. Organic carbon sources (routinely 10 mM sodium malate, sodium succinate, sodium pyruvate, or sodium acetate) and, for oxygen-sensitive strains, 0.5 g sodium ascorbate or 0.25 g thioglycolate are added separately. The initial pH is adjusted to 6.9. Vitamin solution VA contains in 100 ml of double distilled water: biotin, 10 mg; niacin amide, 35 mg; thiamine dichloride, 30 mg; *p*-aminobenzoic acid, 20 mg; pyridoxal hydrochloride, 10 mg; calcium pantothenate, 10 mg; and vitamin B₁₂, 5 mg.

The trace element solution SLA has the following composition: FeCl₂·4H₂O, 1.8 g; CoCl₂·6H₂O, 250 mg; NiCl₂·6H₂O, 10 mg; CuCl₂·5H₂O, 10 mg; MnCl₂·4H₂O, 70 mg; ZnCl₂, 100 mg; H₃BO₃, 500 mg; Na₂MoO₄·2H₂O, 30 mg; and Na₂SeO₃·5H₂O, 10 mg. These components are dissolved in 1 liter of double distilled water. The pH of the solution is adjusted with HCl to 2–3.

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen or at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOSPIRILLUM* FROM OTHER GENERA

A number of chemotaxonomic properties distinguish *Rhodospirillum* species from other spiral-shaped Purple Nonsulfur Bacteria (PNSB). They differ from *Phaeospirillum* species, their closest relatives, in major quinone components and cytochrome *c* structure. Large-type cytochromes c_2 are present in *R. rubrum* and *R. photometricum*, whereas small-type cytochromes c_2 were found in *Phaeospirillum* species (Ambler et al., 1979). Major differentiating properties for phototrophic bacteria in the *Rhodospirillaceae* are shown in Table BXII.α.1. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

TAXONOMIC COMMENTS

Traditionally all spiral-shaped phototrophic PNSB have been assigned to the genus *Rhodospirillum* (Pfennig and Trüper, 1974). Recognition of the large amount of chemotaxonomic and phylogenetic diversity in the PNSB, and their presence in different groups of the *Proteobacteria*, initially led to the taxonomic reclassification of those species belonging to the *Betaproteobacteria*. "*Rhodospirillum tenue*" was assigned to *Rhodocyclus tenuis* (Imhoff et al., 1984) and this reclassification was later supported by 16S rDNA sequence analyses (Hiraishi et al., 1991a). After this reclassification, all species of the genus *Rhodospirillum* were *Alphaproteobacteria*, though the group remained very heterogeneous in phenotypic properties and genetic relationships.

The description of new species, assigned to the genus *Rhodospirillum*, based merely on their spiral shape, continued until recently, although most of them were quite distinct from *Rhodospirillum rubrum*, the type species of this genus. Four halophilic species were classified together with several freshwater species of the genus *Rhodospirillum*. In addition, *Rhodospirillum centenum* (Favinger et al., 1989) was assigned to this genus, though significant differences from *Rhodospirillum rubrum* had been stated in the species description. More recently, the great genetic distance among the spiral-shaped PNSB has been recognized in several proposals. *Rhodospirillum centenum* was transferred to a new genus as *Rhodocista centenaria* (Kawasaki et al., 1992). Another new spiral-shaped species, *Rhodospira trueperi*, was assigned to a new genus based on significant phenotypic and genotypic differences from *Rhodospirillum rubrum* and other known PNSB (Pfennig et al., 1997).

The anticipated heterogeneity of the genus *Rhodospirillum* became clearly apparent with the 16S rDNA sequence information of most of the known species (Kawasaki et al., 1993b; Imhoff et al., 1998), and these data implied that the spiral-shaped *Alphaproteobacteria* are phylogenetically quite distantly related to each

other and do not warrant classification in one and the same genus. Therefore, a reclassification of the spiral-shaped phototrophic *Alphaproteobacteria*, based on distinct phenotypic properties and 16S rDNA sequence similarities, has been proposed (Imhoff et al., 1998; see Table BXII.α.1 and Fig. 1 [p. 124] of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A).

Major quinone components and fatty acid composition, salt requirements, and phylogenetic relationships based on 16S rDNA sequences were considered of primary importance in defining and differentiating these genera. Several phylogenetic lines of salt dependent species were recognized and the salt-dependence was regarded as a genus-specific property (Imhoff et al., 1998). Four of the genera of spiral-shaped phototrophic *Alphaproteobacteria* were defined as salt-dependent and three are freshwater bacteria. Only *R. rubrum* and *R. photometricum* were maintained as species of the genus *Rhodospirillum*. All other species were transferred to the new genera *Phaeospirillum*, *Rhodovibrio*, *Rhodothalassium*, and *Roseospira* (Imhoff et al., 1998) and are considered in the respective chapters of this volume.

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOSPIRILLUM*

Major differentiating properties between *Rhodospirillum* species are shown in Tables BXII.α.1 and BXII.α.2.

TABLE BXII.α.1. Diagnostic properties of the spiral-shaped phototrophic *Alphaproteobacteria*^a

Characteristic	<i>Rhodospirillum rubrum</i>	<i>Rhodospirillum photometricum</i>	<i>Phaeospirillum fulvum</i>	<i>Phaeospirillum molischianum</i>	<i>Rhodocista centuraria</i>	<i>Rhodospira trueperi</i>	<i>Rhodovibrio salinarum</i>	<i>Rhodovibrio sodomensis</i>	<i>Roseospira mediosalina</i>	<i>Roseospirillum parvum</i>
Cell diameter (µm)	0.8–1.0	1.1–1.5	0.5–0.7	0.7–1.0	1.0–2.0	0.6–0.8	0.8–0.9	0.6–0.7	0.8–1.0	0.4–0.6
Internal membrane system	Vesicles	Stacks	Stacks	Stacks	Lamellae	Vesicles	Vesicles	Vesicles	Vesicles	Lamellae
Motility	+	+	+	+	+	+	+	+	+	+
Color	Red	Brown	Brown	Brown	Pink	Beige	Red	Pink	Pink	Pink
Bacteriochlorophyll	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
Growth factors	Biotin	Nicotinamide	<i>p</i> -Aminobenzoic acid	Amino acids	Biotin, B ₁₂	Biotin, thiamine, pantothenate	Cobalamine	Cobalamine	Thiamine, <i>p</i> -aminobenzoic acid, nicotinamide	Cobalamine
Aerobic growth	+	–	–	–	+	–	+	+	+	(+)
Oxidation of sulfide	+	–	–	–	nd	+	–	nd	+	(+)
Salt requirement ^b	None	None	None	None	None	2% (0.5–5)	8–12% (3–24)	12% (6–20)	4–7% (0.5–15)	1–2% (to >6.0%)
Optimal temperature (°C)	30–35	25–30	25–30	30	40–45	25–30	42	35–40	30–35	30
Optimal pH	6.8–7.0	6.5–7.5	7.3	7.3	6.8	7.3–7.5	7.5–8.0	7	7	7.9
Habitat	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water, warm springs	Marine sediments	Saltern	Salt lakes	Salty springs	Marine sediments
Mol% G + C of the DNA	63.8–65.8	64.8–65.8	64.3–65.3	60.5–64.8	69.9	65.7	67.4	66.2–66.6	66.6	71.2
Cytochrome <i>c</i> size	Large	Large	Small	Small	nd	nd	None	None	nd	nd
Major quinones	Q-10, RQ-10	Q-8, RQ-8	Q-9, MK-9	Q-9, MK-9	Q-9	Q-7, MK-7	Q-10, MK-10	nd	nd	nd
Major fatty acids:										
C _{14:0}	2.1	1.0	0.6	0.7	nd	7.5	1.0	nd	nd	nd
C _{16:0}	14.0	25.2	15.1	18.1	nd	27.9	7.4	nd	nd	nd
C _{16:1}	27.1	22.2	25.8	36.5	nd	1.2	0.3	nd	nd	nd
C _{18:0}	1.3	0.4	1.2	0.7	nd	1.2	23.0	nd	nd	nd
C _{18:1}	54.8	51.0	54.5	43.5	nd	60.7	35.2	nd	nd	nd

^aSymbols: +, positive in most strains; –, negative in most strains; (+), weak growth or microaerobic growth only; nd, not determined; Q-7, ubiquinone 7; Q-8, ubiquinone 8; Q-9, ubiquinone 9; Q-10, ubiquinone 10; Q-9/10, ubiquinones 9 and 10; MK-7, menaquinone 7; MK-9, menaquinone 9; MK-10, menaquinone 10; MK-9/10, 10, menaquinones 9 and 10.

^bThe first set of figures indicates the optimum salt concentration, and the second set– the ones in parentheses– indicates the range of salt concentrations tolerated.

List of species of the genus Rhodospirillum

1. **Rhodospirillum rubrum** (Esmarch 1887) Molisch 1907, 25^{AL} (*Spirillum rubrum* Esmarch 1887, 230.)
rub'rum. M.L. neut. adj. *rubrum* red.

Cells are vibrioid shaped to spiral, 0.8–1.0 µm wide; one complete turn of a spiral is 1.5–2.5 µm wide and 7–10 µm long (Fig. BXII.α.1). Internal photosynthetic membranes

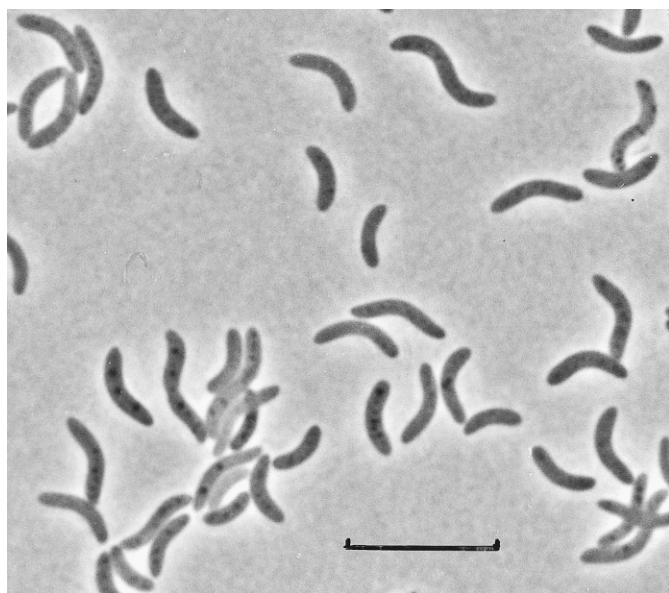


FIGURE BXII.α.1. *Rhodospirillum rubrum* ATCC 11170. Phase-contrast micrograph. Bar = 10 µm. (Courtesy of N. Pfennig).

are of the vesicular type (Fig. BXII.α.2). Anaerobic liquid cultures are pink to deep red, without a brownish tinge, under all conditions. Under oxic conditions, cells are colorless to light pink. Living cells show absorption maxima at 375–377, 510–517, 546–550, 590–595, 807–808, and 881–885 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with geranylgeraniol as major and phytol as minor component) and carotenoids of the spirilloxanthin series with spirilloxanthin as the predominant component.

Cells preferentially grow photoheterotrophically under anoxic conditions in the light with various organic compounds as carbon and electron sources. Photoautotrophic growth occurs with H₂ and sulfide as electron donors. Chemotrophic growth occurs under microoxic to oxic conditions in the dark. Fermentative metabolism with pyruvate under anoxic dark conditions and “oxidant-dependent” anaerobic dark metabolism are also possible. The carbon sources utilized are shown in Table BXII.α.2. In addition, alanine and asparagine are used and some strains use propanol. Ammonia, N₂, several amino acids, and in some strains, nitrate, adenine, guanine, xanthin, and uric acid may be used as nitrogen source. Sulfate can serve as sole sulfur source. Small amounts of yeast extract may be favorable. Biotin is required as a growth factor.

Mesophilic freshwater bacterium with optimum growth at 30–35°C and pH 6.8–7.0 (pH range: 6.0–8.5). Habitat: stagnant and anoxic freshwater habitats that are exposed to the light. Major quinone components are Q-10 and RQ-10.

The mol% G + C of the DNA is: 63.8–65.8 (Bd).

Type strain: ATCC 11170, DSM 467, NCIB 8255.

GenBank accession number (16S rRNA): D30778, M32020.

2. **Rhodospirillum photometricum** Molisch 1907, 24^{AL}
pho.to.me'tri.cum. Gr. n. *phos* light; Gr. adj. *metricus* measuring; M.L. neut. adj. *photometricum* light measuring.

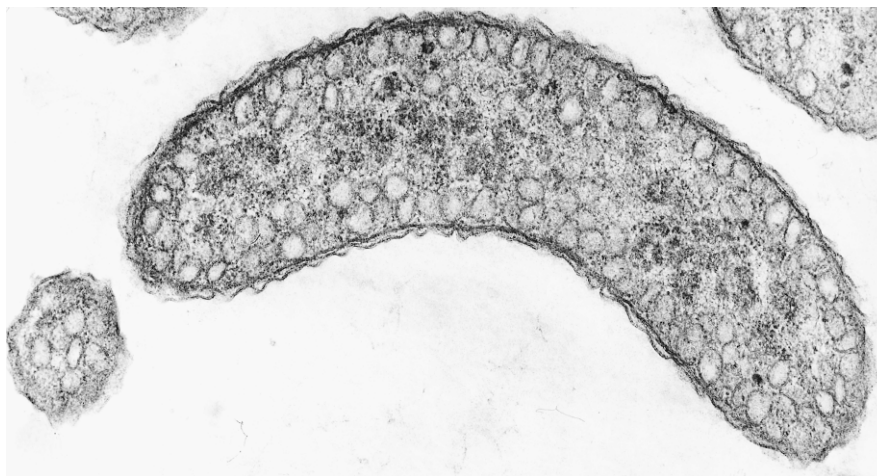
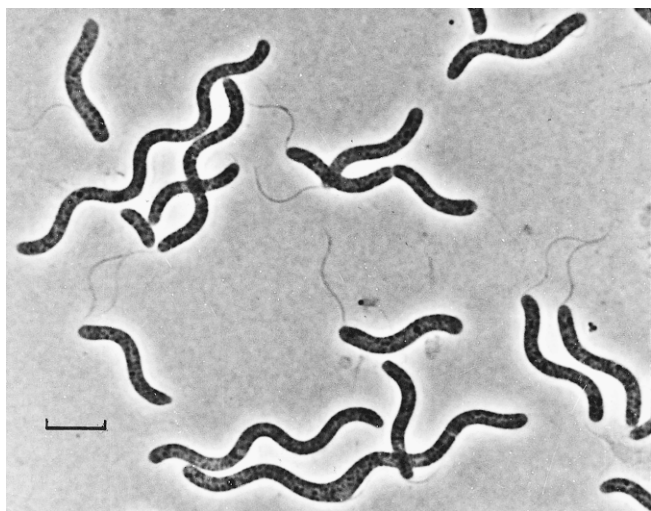


FIGURE BXII.α.2. *Rhodospirillum rubrum* strain FR1 grown anaerobically in the light. Note the vesicular structure of the intracytoplasmic membrane system. × 51,000. (Courtesy of G. Drews and R. Ladwig).

TABLE BXII.α.2. Carbon sources and electron donors used by species of the anoxygenic phototrophic bacteria belonging to the family *Rhodospirillaceae*^a

Source/donor	<i>Rhodospirillum rubrum</i>	<i>Rhodospirillum photometricum</i>	<i>Phaeospirillum fulvum</i>	<i>Phaeospirillum molischianum</i>	<i>Rhodocista centenaria</i>	<i>Rhodospira trueperi</i>	<i>Roseospira mediosalina</i>	<i>Roseospirillum parvum</i>
<i>Carbon sources:</i>								
Acetate	+	+	+	+	+	+	+	+
Arginine	+	—	—	—	nd	nd	—	nd
Aspartate	+	+ / —	+ / —	+ / —	+	nd	+	nd
Benzoate	—	—	+	—	nd	nd	—	nd
Butyrate	+	+	+	+	+	+	+	+
Caproate	+	—	+	+	+	nd	nd	nd
Caprylate	nd	—	+	+	+	nd	—	nd
Citrate	—	—	—	—	nd	nd	—	nd
Ethanol	+	+	+	+	nd	—	—	nd
Formate	—	—	nd	nd	nd	nd	—	nd
Fructose	+ / —	+	—	—	—	nd	—	+
Fumarate	+	+	+	+	—	+	+	+
Glucose	—	+	+ / —	—	—	nd	—	nd
Glutamate	+	—	nd	nd	+	nd	+	+
Glycerol	—	+	—	—	nd	nd	—	nd
Glycolate	nd	+	nd	—	nd	nd	—	nd
Lactate	+	+	—	+ / —	+	+	+	+
Malate	+	+	+	+	—	+	+	+
Mannitol	—	+	—	—	nd	nd	—	nd
Methanol	+ / —	—	+ / —	—	nd	nd	—	nd
Pelargonate	nd	+ / —	+	+	nd	nd	nd	nd
Propionate	+	+ / —	+	+	nd	+	+	+
Pyruvate	+	+	+	+	+	+	+	+
Succinate	+	+	+	+	—	+	+	+
Tartrate	—	—	nd	—	nd	nd	—	nd
Valerate	+	+	+	+	+	+	—	+
<i>Electron donors:</i>								
Hydrogen	+	+	nd	nd	nd	nd	nd	nd
Sulfide	+	—	—	—	nd	+	+	+
Sulfur	—	—	—	—	nd	—	—	nd
Thiosulfate	—	—	—	—	nd	—	—	+

^aSymbols: +, positive in most strains; —, negative in most strains; + / —, variable in different strains; nd, not determined.**FIGURE BXII.α.3.** *Rhodospirillum photometricum* NTHC 132, cultured on malate-yeast extract medium. Phase-contrast micrograph. Bar = 10 μm. (Courtesy of N. Pfennig).

Cells are spirals, 1.1–1.5 μm wide; one complete turn of a spiral is 2.5–4 μm wide and 4–7 μm long; cells 14–30 μm long are common (Fig. BXII.α.3). Internal photosynthetic membranes consist of several lamellar stacks forming a sharp angle with the cytoplasmic membrane. Anaerobic liquid cultures are brown-orange to brown-red or dark brown.

Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol and in some strains also with geranylgeraniol) and carotenoids of the spirilloxanthin series. Spirilloxanthin is lacking, but the biosynthetic precursors lycopene and rhodopin are present as major components.

Cells preferentially grow photoheterotrophically under anoxic conditions in the light with various organic compounds as carbon and electron sources. Chemotrophic growth at very low oxygen tensions, but not under oxic growth conditions, is possible. Under microoxic conditions cells are fully pigmented. Carbon sources utilized are shown in Table BXII.α.2. In addition, asparagine, maltose, sucrose, raffinose, adonitol, and dulcitol are used. Not used are cyclohexane carboxylate, mannose, galactose, xylose, and inositol. Nitrogen sources utilized are ammonia, N₂, alanine, glutamate, and asparagine; not utilized are nitrate, urea, and arginine. Sulfate, thiosulfate, cysteine, thioglycolate, and, at low concentrations, sulfide can be used as sulfur sources. Ascorbic acid may be required as a reductant. Nicotinic acid is required as a growth factor.

Mesophilic freshwater bacterium with optimum growth at 25–30°C and pH 6.5–7.5. Habitat: stagnant and anoxic freshwater habitats that are exposed to the light. Major quinone components are Q-8 and RQ-8.

The mol% G + C of the DNA is: 64.8–65.8 (Bd) and 63 (T_m).

Type strain: ATCC 49918, DSM 122, NTHC 132.

GenBank accession number (16S rRNA): AJ222662.

Genus II. Azospirillum Tarrand, Krieg and Döbereiner 1979, 79^{AL} (Effective publication: Tarrand, Krieg and Döbereiner 1978, 1978) emend. Falk, Döbereiner, Johnson and Krieg 1985, 117

JOSÉ IVO BALDANI, NOEL R. KRIEG, VERA LÚCIA DIVAN BALDANI, ANTON HARTMANN AND JOHANNA DÖBEREINER

A.zo.spi.ril' lum. Fr. n. *azote* nitrogen; Gr. n. *spira* a spiral; M.L. dim. neut. n. *spirillum* a small spiral; *Azospirillum* a small nitrogen spiral.

Plump, slightly-curved and straight rods, 0.6–1.7 × 2.1–3.8 µm, often with pointed ends. Intracellular granules of poly-β-hydroxybutyrate are present. Enlarged, pleomorphic forms may occur in old, alkaline cultures, under conditions of excess oxygen or other stress. Gram negative to Gram variable. **Motile in liquid media by a single polar flagellum**; on solid media at 30°C, numerous lateral flagella of shorter wavelength may also be formed. **Nitrogen fixers, exhibiting N₂-dependent growth under microaerobic conditions.** Grow well under an air atmosphere in the presence of a source of fixed nitrogen such as an ammonium or glutamate salts. Cells previously grown in presence of an inorganic nitrogen source may fix nitrogen in air provided that all added nitrogen is exhausted and nitrogenase is derepressed. Possess mainly a respiratory type of metabolism with oxygen and, with some strains, nitrate or nitrite as the terminal electron acceptor. Fermentative metabolism may also occur. **Under severe oxygen limitation, some strains may dissimilate nitrate to nitrite or to nitrous oxide and nitrogen gas.** Optimal temperature for growth varies from 33 to 41°C and pH from 5.5 to 7.5. Some strains may grow and form light or dark pink colonies, often wrinkled and non-slimy, on potato agar. Oxidase positive. Chemoorganotrophic; some strains are facultative hydrogen autotrophs. **Grow well on salts of organic acids such as malate, succinate, lactate or pyruvate. D-fructose and certain carbohydrates may also serve as carbon sources.** Some species require biotin. Growth in presence of 3% NaCl has been observed for some species. **Occur free-living in the soil or associated with the roots, stems, leaves, and seeds** mainly of cereals and forage grasses, although they have also been isolated from coconut plants, vegetables, fruits, legume, and tuber plants. May also be found in freshwater lakes. Root nodules are not induced.

The mol% G + C of the DNA is: 64–71.

Type species: *Azospirillum lipoferum* (Beijerinck 1925) Tarrand, Krieg and Döbereiner 1979, 79 (Effective publication: Tarrand, Krieg and Döbereiner 1978, 1978) (*Spirillum lipoferum* Beijerinck 1925, 353.)

FURTHER DESCRIPTIVE INFORMATION

Morphology In complex media such as MPSS broth¹, *A. lipoferum* and *A. brasilense* grow as plump, slightly curved rods and straight cells having a diameter of ~1.0 µm. Many of the cells have pointed ends. In semisolid nitrogen-free malate (NFB) medium², *A. lipoferum* develops predominantly into pleomorphic

cells within 48 h (Fig. BXII.α.4), in contrast to *A. brasilense*, which retains mainly the vibrioid form. *A. lipoferum* grows as elongated cells (1.4–1.7 µm × 5 to over 30 µm long), which are nonmotile and have an S shape or helical shape (Fig. BXII.α.4). These forms eventually seem to fragment into shorter ovoid forms, many of which become very large and rounded and may contain several cells filled with phase-refractile granules (probably poly-β-hydroxybutyrate). Alkalinization of the malate medium, due to oxidation of the malate, may be related to development of pleomorphism in *A. lipoferum*. Pleomorphism fails to occur when the organisms are cultured in semi-solid nitrogen-free D-glucose medium, which does not become alkaline (Fig. BXII.α.5).

In semi-solid nitrogen-free malate medium, *A. brasilense* grows mainly as motile, vibrioid cells. Nonmotile, enlarged, pleomorphic forms (C forms) may also occur, especially in older cultures (Eskew et al., 1977; Tarrand et al., 1978), on the surface of nitrogen-free agar media (Berg et al., 1980), in association with plant callus cultures (Berg et al., 1979), or in association with the roots of grass seedlings (Umali-Garcia et al., 1980). A capsule is formed external to the outer wall membrane of C forms (Fig. BXII.α.6) and may be a protective mechanism against unfavorable levels of oxygen under nitrogen-fixing conditions (Berg et al., 1980). Very large, rounded forms containing several cells may be produced (Fig. BXII.α.6). The ultrastructure of the C forms indicates little similarity to the cysts of *Azotobacter*. Lamm and Neyra (1981) have reported that strains of *A. brasilense* and *A. lipoferum* enriched in C forms exhibit a resistance to desiccation and temperature not found in cultures lacking these forms. Cyst forms of *A. brasilense* could be physiologically active in the wheat rhizosphere, as shown by a high level of 16S rRNA-directed oligonucleotide binding (Assmus et al., 1995). Cysts are capable of fixing nitrogen in the absence of an exogenous carbon source (Okon and Itzigsohn, 1992). *A. amazonense* does not develop pleomorphic cells when grown in the semi-solid LGI medium because the pH is maintained around 6. Even when a very poor subsurface pellicle is formed in semi-solid NFB medium (this species is sensitive to alkaline pH), no such cells are observed. In LGI medium, cells remain motile and vibrioid. However, cultures maintained two to three weeks on the bench develop agglomerated cells like cysts due to the dryness of the medium.

Pleomorphic cells have been observed for the species *A. halopraeferens*, *A. irakense*, and *A. largimobile*, depending on the growth conditions. In the case of *A. halopraeferens* this phenomenon is observed in older and very alkaline conditions whereas very long cells (30 µm) are observed when *A. irakense* is grown in nutrient broth (Fig. BXII.α.7). On the other hand, *A. largimobile* forms multicellular conglomerates of nonmotile cells mainly under unfavorable conditions. The most recently described species, *A. doebereineriae*, also develops pleomorphic cells in semi-solid NFB medium.

Encapsulation may be related to resistance to Gram-decolorization exhibited by a small proportion of cells when cultured on MPSS agar at 37°C for 48–72 h. Gram-variability appears to be more pronounced with *A. brasilense* than with *A. lipoferum*.

1. MPSS broth (g/l): peptone (Difco), 5.0; succinic acid (free acid), 1.0; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 1.0; FeCl₃·6H₂O, 0.002; MnSO₄·H₂O, 0.002; pH adjusted to 7.0 with KOH. For solid media add 15.0 g/l agar.

2. NFB medium (g/l): L-malic acid, 5.0; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.02; trace element solution (Na₂MoO₄·2H₂O, 0.2 g; MnSO₄·H₂O, 0.235 g; H₃BO₃, 0.28 g; CuSO₄·5H₂O, 0.008 g; ZnSO₄·7H₂O, 0.024 g; distilled water, 1000 ml), 2.0 ml; bromthymol blue (0.5% aqueous solution [dissolve in 0.2 N KOH]), 2.0 ml; Fe EDTA (1.64% solution), 4.0 ml; vitamin solution (biotin, 0.01 g; pyridoxin, 0.02 g; distilled water, 1000 ml), 1.0 ml; KOH, 4.0; pH adjusted to 6.8 with KOH. For a semi-solid medium add 1.75 g/l agar; for a solid medium add 15.0 g/l agar.



FIGURE BXII.α.4. *Azospirillum lipoferum* ATCC 29707 cultured in semi-solid, nitrogen-free, malate medium at 37°C for 48 h, showing characteristic elongated S-shaped forms and enlarged ovoid forms. Phase contrast microscopy. Bar = 5 μm.

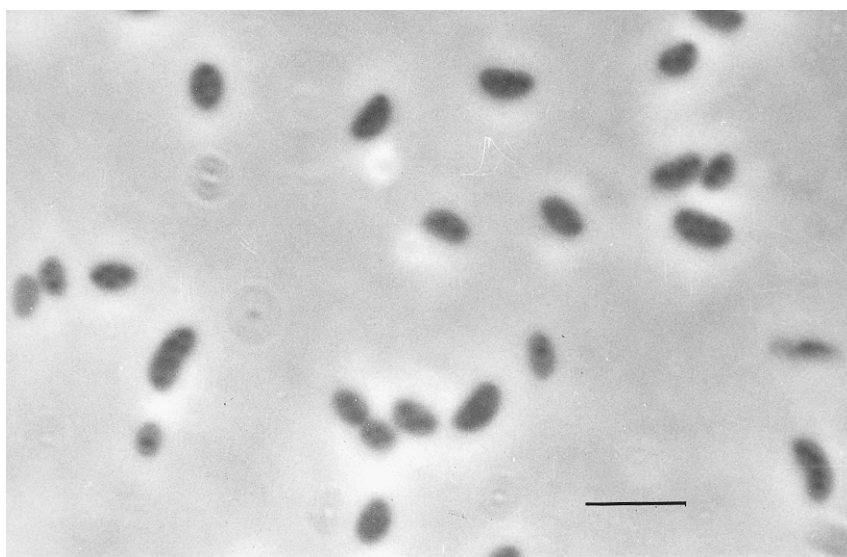


FIGURE BXII.α.5. *Azospirillum lipoferum* ATCC 29707 cultured in semi-solid, nitrogen-free, malate medium at 37°C for 48 h, showing characteristic vibrioid forms. Phase contrast microscopy. Bar = 5 μm.

Cultures grown in MPSS broth appear not to contain encapsulated forms, at least in young cultures, and the cells stain uniformly Gram negative. So far, no Gram variability has been observed for the other *Azospirillum* species.

Poly-β-hydroxybutyrate (PHB) may constitute from 25 to 50% of the dry weight of cells cultured in nitrogen-free media. In cells cultured with an ammonium salt as the nitrogen source, the polymer constitutes only 0.5–1.0% of the cell weight (Okon et al., 1976). However, high amounts of poly-β-hydroxybutyrate are accumulated in cells of *A. brasilense* strain Cd grown in a high C:N medium using D-fructose and ammonium chloride as C and N source (Burdman et al., 1998). The amount of PHB also increases under various stress conditions such as toxic metals and water stress, and this accumulation is correlated with cyst and

floc formation where it accounts for up to 65% of the total dry weight (Olubayi et al., 1998). Cells rich in PHB survive better and promote positive effect on cereals (Assmus et al., 1995; Fallik and Okon, 1996). It has been suggested that inoculants generated from flocculated cells are more advantageous because they seem to be more efficient than those originating from nonflocculated cells, and in addition they can be produced on a large scale and easily separated from the growth medium (Neyra et al., 1995; Burdman et al., 1998). A high amount of PHB has been observed in *A. amazonense* species grown under nitrogen-fixing conditions (Fig. BXII.α.8) but not with an inorganic nitrogen source. Intracellular granules (probably PHB) were also observed in old cells of *A. doebereineriae*.

Azospirilla possess a single polar flagellum when grown in

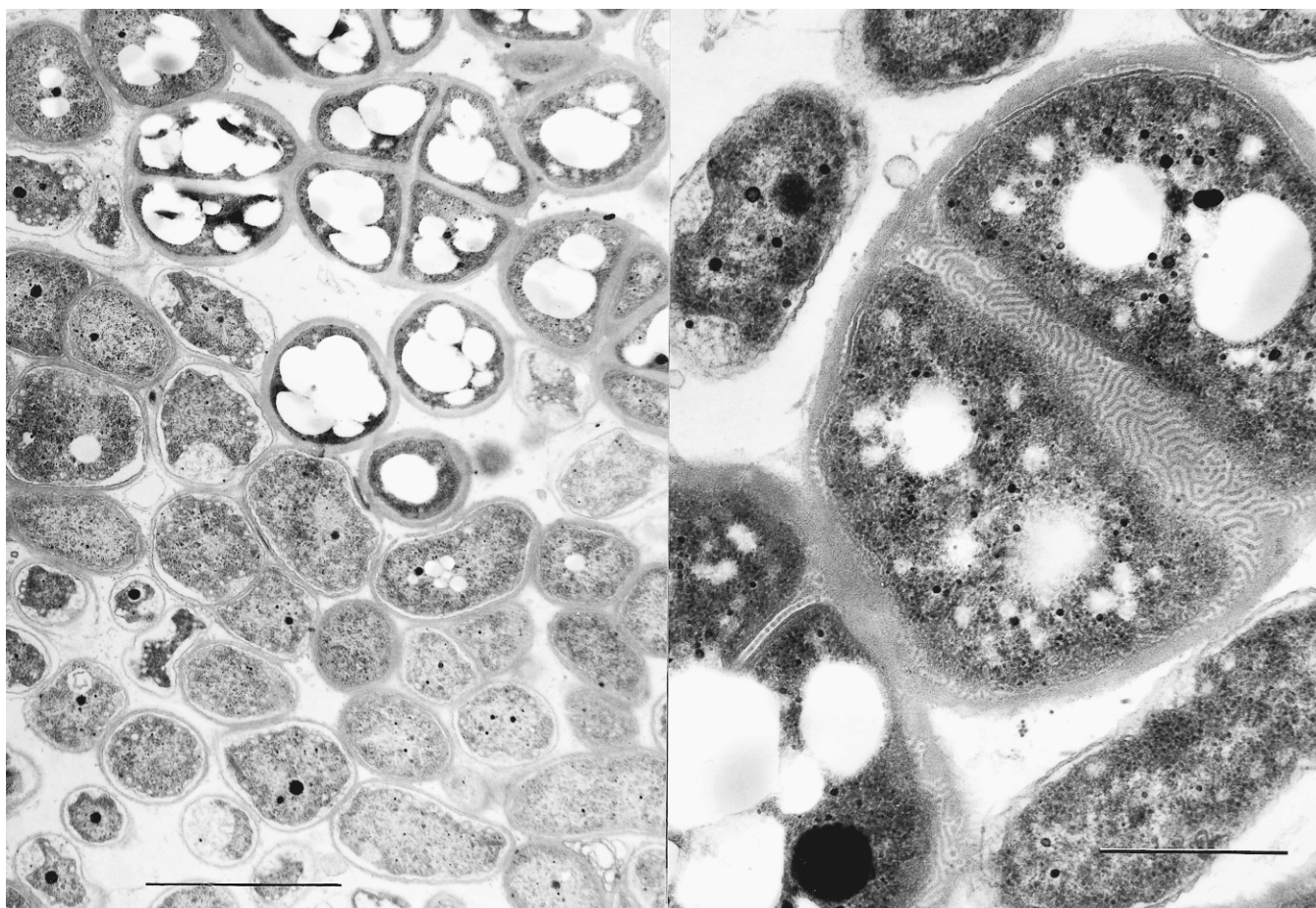


FIGURE BXII.α.6. Ultrastructure of vibrioid and encapsulated (C) forms of *Azospirillum brasilense* ATCC 29145 grown in association with a plant callus (sugarcane). *Left*: gradient of vibrioid forms (*lower*) to C forms (*upper*). Multicellular C forms filled with poly-β-hydroxybutyrate granules (white areas) are seen at the top. Bar = 1.5 μm. *Right*: comparative fine structure of vibrioid forms and C forms. Bar = 0.5 μm. (Reproduced with permission from R.H. Berg et al., *Protoplasma* 101: 143–163, 1979, ©Springer-Verlag, Vienna.)

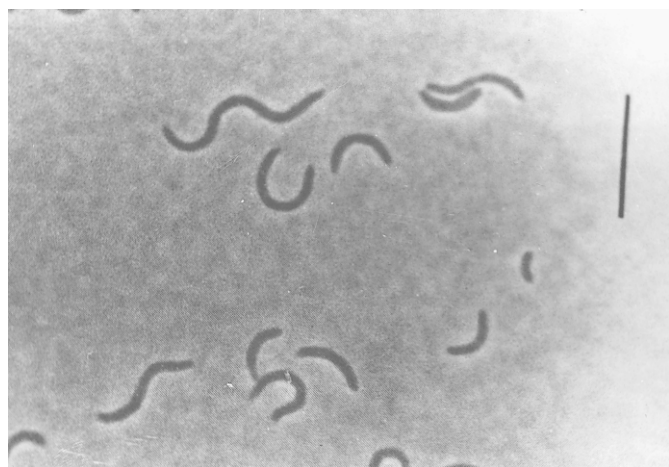


FIGURE BXII.α.7. Phase contrast microscopic examination of *Azospirillum irakense* strain KBC1 grown at 30°C for 20 h in complete nutrient broth showing the curved rods or S-shaped forms. Bar = 10 μm. (Reproduced with permission from K.M. Khammas et al., *Research in Microbiology* 140: 679–693, 1989, Editions Scientifiques et Medicales, ©Elsevier, Paris.)



FIGURE BXII.α.8. Transmission electron micrograph of *Azospirillum amazonense* strain ATCC 35119 grown in semi-solid LGI medium for 24 h at 30°C. Note the presence of poly-β-hydroxybutyrate granules inside the cell. (~× 13,000). (Reproduced with permission from F.M. Magalhães et al., *Anais da Academia Brasileira de Ciencias* 55: 417–430, 1983, ©Academia Brasileira de Ciencias, Rio de Janeiro.)

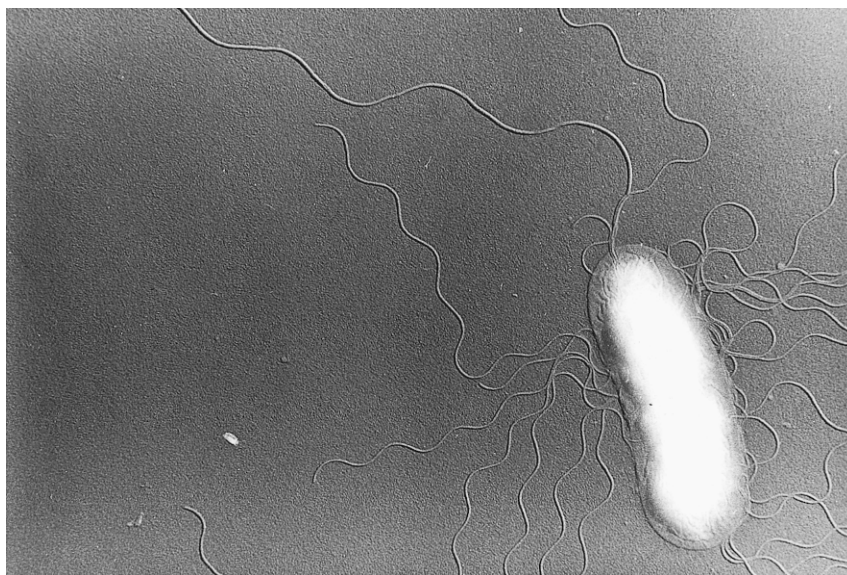


FIGURE BXII.α.9. Electron micrograph of *Azospirillum brasilense* ATCC 29145 cultured on MPSS agar at 30°C for 24 h. Both the single polar flagellum and the numerous lateral flagella can be seen. Shadowed with tungsten oxide. ($\sim \times 15,000$).

liquid media. However, when cultured on MPSS agar at 30°C numerous lateral flagella in addition to the polar flagellum are formed in the species *A. lipoferum* and *A. brasilense* (see Fig. BXII.α.9). Lateral flagella are also observed in strains of *A. irakense* grown in nutrient broth but not in *A. amazonense*. The polar flagellum appears to be thicker than the lateral flagella and has a longer wavelength. *A. largimobile* cells have a mixed flagellation with a single polar flagellum and 1–10 distinct lateral flagella of different thickness and wavelength when grown in LWA medium. The polar flagellum is not only involved in aero- and chemotaxis (Grishanin et al., 1991), but also in the first adsorption step in the attachment process of *A. brasilense* to wheat roots (Michiels et al., 1991). The function of the lateral flagella is unknown, although a mutant of *A. brasilense* strain Cd lacking both polar and lateral flagella developed a stronger flocculation of cells when grown in a high C:N medium containing D-fructose and ammonium chloride as C and N source (Burdman et al., 1998).

Cultural characteristics On BMS agar³ after 1–2 weeks of incubation at 33–35°C, colonies of *A. brasilense* and *A. lipoferum* are pink, opaque, irregular or round, often wrinkled, and typically have umbonate elevations (Döbereiner and Baldani, 1979). Pigmentation is best on BMS agar incubated under light. Certain strains and variants of *A. brasilense* form colonies that have a very deep pink color (Eskew et al., 1977; Tarrand et al., 1978). In one such strain (ATCC 29729), this intense color is attributable to the formation of several carotenoid pigments that occur only

under aerobic conditions and may be related to protection of the nitrogenase from oxidative damage (Nur et al., 1981). This hypothesis was supported by the study of Hartmann and Hurek (1988) who compared carotenoid-overproducing mutants of *A. brasilense* strain Sp7 and carotenoid-negative mutants of strain Cd. Carotenoid overproducing mutants exhibited slightly higher oxygen tolerance. On the other hand, no pigment has been observed for strains of the other *Azospirillum* species.

In nitrogen-free media, nitrogen fixation occurs only under microaerobic conditions ($pO_2 \approx 0.003$ atm), due to lack of oxygen protection mechanisms for the nitrogenase. In liquid nitrogen-free media, there is no growth or nitrogen fixation under an air atmosphere except when cells are previously grown in the presence of an exogenous nitrogen source such as glutamate or ammonium chloride (Pedrosa and Yates, 1984; Baldani et al., 1986a). In this case, strains are grown in conical flasks (250 ml) with 100 ml liquid NFbHP⁴ medium containing a nitrogen source at 32°C and 120 rpm. Derepressed cells are obtained when the culture reaches an OD_{550} of approximately 1.0. This is the easiest way to obtain nitrogenase-derepressed cells for physiological and biochemical studies, especially when a large number of strains are to be assayed. The fastest growth rates are obtained in continuous or batch cultures when the oxygen input is in equilibrium with bacterial respiration. Depending on the cell density, a gas mixture containing from 1 to 25% oxygen can be bubbled through the culture to achieve this. A simpler way to obtain N_2 -dependent growth is by culturing the organisms in semi-solid nitrogen-free media incubated under an air atmosphere. Here, growth is initiated as a thin veil or disc-like pellicle several mil-

3. BMS agar: washed, peeled, sliced potatoes, 200 g; L-malic acid, 2.5 g; KOH, 2.0 g; raw cane sugar, 2.6 g; vitamin solution (biotin, 0.01 g; pyridoxin, 0.02 g; distilled water, 1000 ml), 1.0 ml; bromthymol blue (0.5% alcoholic solution), 2 drops; agar, 15.0 g. The potatoes are placed in a gauze bag, boiled in 1 liter of water for 30 min, then filtered through cotton, saving the filtrate. The malic acid is dissolved in 50 ml of water and the bromthymol blue added. KOH is added until the malic solution is green (pH 7.0). This solution, together with the cane sugar, vitamins and agar, is added to the potato filtrate. The final volume is made up to 1 liter with distilled water. The medium is boiled to dissolve the agar, then sterilized by autoclaving.

4. NFbHP medium (g/l): KH_2PO_4 , 4.0; K_2HPO_4 , 6.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; Nitritoltriacetic acid 0.056; $FeSO_4 \cdot 7H_2O$, 0.02; sodium lactate, 5.0; biotin, 0.0001; $Na_2MoO_4 \cdot 2H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 0.00235; H_3BO_3 , 0.0028; $CuSO_4 \cdot 5H_2O$, 0.00008; $ZnSO_4 \cdot 7H_2O$, 0.00024. Phosphate solutions were autoclaved separately and left to cool before adding to the medium. The volume is completed to 1000 ml and the final pH is 6.8. NH_4Cl is added to a concentration of 20 mM when liquid medium is used. For solid medium add 12 g/l of agar before autoclaving.

limeters or 1 cm below the surface of the medium at a point where the rate of diffusion of oxygen into the medium corresponds to the respiration rate of the organisms so that no excess oxygen remains in solution. As the bacteria multiply, the disc of growth migrates closer to the surface until finally it is just below the surface. The time taken for the veil to move to the surface depends on the *Azospirillum* species. In the case of *A. lipoferum*, *A. brasilense*, and *A. irakense*, the pellicle reaches the surface 30–42 h after incubation, whereas with *A. amazonense*, *A. halopraeferens*, and *A. doebereineriae* it takes around 3–4 days. Okon et al. (1980) have found a similar veil or disc formation even in media containing a source of fixed nitrogen, suggesting that the organisms may prefer microaerobic conditions even when not fixing nitrogen. Under these conditions, the growth is much faster. It has been shown that cells grown in semi-solid NFB or LGI medium containing nitrate can fix nitrogen provided that all accumulated nitrite is reassimilated by the cells and the nitrogenase is derepressed (Magalhães et al., 1983; Baldani et al., 1986a). The optimum level of dissolved oxygen for nitrogenase activity was 0.2 kPa oxygen in *A. brasilense*, *A. lipoferum*, and *A. amazonense* (Hartmann et al., 1985). At transiently increased oxygen levels, a reversible inhibition (switch off) of nitrogenase activity occurred, which was accompanied by a reversible covalent modification of the dinitrogenase reductase of *A. brasilense* and *A. lipoferum* (Hartmann and Burris, 1987). Recently, Vande Broek et al. (1996) demonstrated that *A. brasilense* is slightly more oxygen-sensitive than *A. irakense*, based on the maximum oxygen concentration at which activation of an *A. brasilense* *nifH*–*gusA* fusion and acetylene reduction was still observed under the experimental conditions. Comparison of *A. amazonense* and *A. brasilense* grown in nitrogen-free media in batch cultures seemed to show that the first species is much more oxygen sensitive than the latter (Magalhães et al., 1983). However, measurements in a well-mixed chamber where respiration rates, ARA, and dissolved O₂ were controlled showed that nitrogen fixation and derepression of the nitrogenase of *A. amazonense* is tolerant of a somewhat higher pO₂ than those of *A. lipoferum* and *A. brasilense* (Hartmann et al., 1985). This factor seems to be due to the presence of an oxygen-tolerant hydrogenase in *A. amazonense* (Fu and Knowles, 1989).

When supplied with a source of fixed nitrogen, such as an ammonium salt or nitrate, azospirilla can grow under aerobic conditions. Nitrate is assimilated by an assimilatory nitrate reductase (Neyra and van Berkum, 1977). Azospirilla reduce nitrate to nitrite either by an aerobic assimilatory pathway or an anaerobic dissimilatory or respiratory pathway. A group of *A. amazonense* strains (called *nir*⁺⁺) assimilates nitrite much faster than most strains of the species (called *nir*⁺). Approximately half of the strains of *A. lipoferum* and *A. brasilense* so far isolated can dissimilate nitrite further to nitrous oxide and nitrogen gas (Neyra et al., 1977). Maximum dissimilatory nitrite reductase activity of *A. brasilense* has been observed to occur at high pH values between 7.6 and 8.5 and only under anaerobic conditions (Zimmer et al., 1984). Denitrification has not been observed among strains of *A. amazonense*, *A. irakense*, and *A. largimobile* but it was detected in all strains of *A. halopraeferens*. Nitrite reductase (*nir*) is the key enzyme for denitrification in *Azospirillum* (Magalhães et al., 1978). Plant roots were shown to be mostly infected by non-denitrifying strains (*nir*[−]) of *A. lipoferum* and *A. brasilense*. Most of the roots infected by *A. amazonense* strains belong to the *nir*⁺⁺ group. Chlorate-resistant spontaneous mutants lacking the dissimilatory nitrate reductase (*nir*[−] mutants) and/or the nitrite

reductase (*nir*[−] mutants) have been isolated from *A. lipoferum* and *A. brasilense* species. Nitrogenase activity is retained by such mutants and proceeds even in the presence of 10 mM nitrate. A comparison of several nitrate reductase negative (*nir*[−]) mutants of *A. brasilense* strain Sp245 with the wild type carried out in a monoxenic wheat test tube experiment demonstrated the role of nitrate reductase in nitrate assimilation by wheat plants (Ferreira et al., 1987).

Steady-state cultures of denitrifying strains of *A. brasilense* have been obtained under anaerobic conditions in malate containing 100 mM nitrate. When the level of nitrate is lowered to 20 mM, nitrogenase activity occurs but growth is severely decreased (Nelson and Knowles, 1978). Anaerobic suspensions of nitrogenase-containing cells in NFB medium containing 10 mM nitrate exhibit nitrate-dependent nitrogenase activity after 30–60 min, but no growth occurs (Neyra and van Berkum, 1977; Scott et al., 1979); during this period nitrite is accumulated in the medium. A transitory, nitrate-dependent acetylene reduction has been observed in *A. lipoferum* and *A. brasilense* in nitrogen-fixing but not in NO₃[−]-grown cultures (Bothe et al., 1981).

In peptone-based media, *A. lipoferum* and *A. brasilense* grow abundantly under anaerobic conditions due to the denitrification process, with the nitrate being reduced to nitrite or to nitrous oxide and nitrogen gas. NO₃[−]-dependent anaerobic growth has not yet been shown for *A. amazonense*, *A. halopraeferens*, *A. irakense*, and *A. largimobile*. Other compounds such as nitrite, nitrous oxide, and dimethylsulfoxide (DMSO) can also be used as alternative electron acceptors by *A. lipoferum* and *A. brasilense* strains when NH₄⁺ is supplied as a nitrogen source (Döbereiner, 1992a; Hartmann and Zimmer, 1994).

Low concentrations of ammonium completely inhibit nitrogenase activity in *A. lipoferum* and *A. brasilense* (Pedrosa and Yates, 1984; Hartmann et al., 1986). This mechanism, known as “NH₄⁺ switch on/off”, which involves reversible ADP-ribosylation of the *nifH*-protein (Fu et al., 1989), has not been observed in *A. amazonense*. In *A. amazonense*, a less effective, noncovalent, reversible inhibition of nitrogenase activity by ammonium chloride occurs. Assimilation of ammonium in azospirilla is mediated by the GS-GOGAT pathway. Glutamate dehydrogenase (GDH), glutamate-oxaloacetate-aminotransferase (GOT), and glutamate-pyruvate-aminotransferase (GTP) also play a role when a high level of ammonium or glutamate is present. *A. lipoferum* and *A. amazonense* exhibit high activities of these enzymes and are able to fix nitrogen using glutamate as carbon and nitrogen source when supplied at low concentrations (1–2 mM), whereas *A. brasilense* and *A. halopraeferens* fix nitrogen in the presence of higher levels of glutamate (20 mM) due to the low rate of glutamate assimilation (Hartmann and Zimmer, 1994). While the amino acids aspartate and histidine showed the same differential effect as glutamate on the nitrogenase activity of azospirilla, the amino acids glutamine, asparagine, arginine, and alanine inhibited nitrogenase effectively (Hartmann et al., 1988).

Azospirillum mutants able to fix nitrogen in the presence of external ammonium sources have been developed. Prototrophic mutants of *A. brasilense* resistant to ethylenediamine (EDA) and able to excrete NH₄⁺ were selected. Studies carried out under gnotobiotic conditions showed that these mutants support the growth of wheat plants better than the wild type strains (Christiansen-Weniger and Van Veen, 1991).

Nitrogenase complex The nitrogenase system of *Azospirillum* consists of two components: the Mo-Fe protein and the Fe protein (Ludden et al., 1978). An activating factor for the Fe protein

(Mn^{2+} -dependent activation process) is required for *A. lipoferum* and *A. brasilense* (Ludden et al., 1978; Pedrosa and Yates, 1984) but not for *A. amazonense* (Song et al., 1985). So far, no studies have been carried out for the other *Azospirillum* species. The activating factor is interchangeable with that from *Rhodospirillum rubrum*. An alternative nitrogenase system 3 (Fe-nitrogenase) in addition to the Mo-nitrogenase has been suggested in *A. brasilense* strain Cd (Chakraborty and Samaddar, 1995) but it lacks confirmation. A highly purified dinitrogenase (MoFe-protein) in an active form was obtained from *A. amazonense* (Song et al., 1985) and shown to be a tetramer of 210 kDa with subunits ($\alpha_2\beta_2$) having molecular weights of 50 and 55 kDa, respectively. The dinitrogenase reductase (Fe-protein) is a dimer with subunits of 31 and 35 kDa. Similar results were observed with *A. doebereineriae* strains grown under dinitrogen-dependent conditions, as detected by immunoassay of blotted cell extracts against the Fe-protein of *R. rubrum* and *A. vinelandii* antisera (Eckert et al., 2001). The molecular weights of the nitrogenase component peptides from *A. brasilense* strain Sp7 were determined by immunoprecipitation against antisera from *K. pneumoniae* and were estimated to be 60 kDa and 64 kDa for MoFe protein subunits and 33 kDa and 36 kDa for Fe protein subunits (Nair et al., 1983). The nitrogenase system *in vivo* in *A. lipoferum*, *A. brasilense*, and *A. amazonense* does not liberate H_2 because the latter is recycled by a hydrogenase (Berlier and Lespinat, 1980; Chan et al., 1980; Volpon et al., 1981; Pedrosa and Yates, 1984; Fu and Knowles, 1988). No data are yet available for the other species.

Hydrogenase and autotrophy *A. lipoferum* has the potential for H_2 -dependent autotrophic growth under nitrogen-fixing or non-nitrogen-fixing conditions. Strain 208 was able to grow autotrophically under an atmosphere of $\text{H}_2/\text{CO}_2/\text{O}_2/\text{N}_2$ (20:5:2:73) in liquid NFb medium (Sampaio et al., 1981). Both ribulose-1,5-bisphosphate carboxylase (RubPcase) and hydrogenase activities were present and were much higher in autotrophically grown cells than in lactate-grown cells. In contrast to *A. lipoferum*, *A. brasilense* ATCC 29145 showed only marginal growth with H_2 under autotrophic conditions. Málek and Schlegel (1981) found that all of the strains of *A. lipoferum* they tested (including the type strain) were capable of hydrogenase activity and autotrophic growth when the O_2 concentration in the gas atmosphere was less than 2% (v/v). RubPcase and hydrogenase activities were present. In the absence of a source of combined nitrogen, the growth rate was very slow under autotrophic conditions and ceased at a cell concentration of 0.2 g/l. With an ammonium salt present, the growth rate was much faster. None of the strains of *A. brasilense* tested was capable of autotrophic growth. In addition, no reports are yet available on autotrophic growth for the other described species of *Azospirillum*.

Methyлотrophy Both *A. brasilense* ATCC 29145 and *A. lipoferum* Sp 208 grow well on methane, methanol, or formate as sole energy sources (Sampaio et al., 1981). *A. lipoferum* cells derepressed under CH_4 were devoid of RubPcase and hydrogenase activities; however, RubPcase, but not hydrogenase, could be induced by CO_2 in the presence of methane. These results strongly suggest that *A. brasilense* and *A. lipoferum* have potential for methyлотrophic growth and according to Sampaio et al. (1982), these two species could be classified as facultative methyлотrophs. However, these authors were not able to demonstrate ^{14}C -incorporation into *A. brasilense* and *A. lipoferum* biomass. These results have not yet been confirmed by other studies, and there are no reports of methyлотrophy in other *azospirilla* species.

Nutritional characteristics Compounds that can serve as sole carbon and energy sources for N_2 -dependent growth of *A. brasilense*, *A. lipoferum*, *A. halopraeferens*, *A. irakense*, *A. largimobile*, and *A. doebereineriae* include malate, succinate, pyruvate, and lactate. *A. amazonense* strains can also use these carbon sources, but their sensitivity to alkaline pH values inhibits growth and misleading results can be obtained. A highly buffered medium can circumvent the problem, provided that the initial pH is around 6.0. D-Fructose can also be used as a carbon source by all species except *A. irakense*, in which variable results have been observed. D-Glucose is not used by *A. brasilense* and *A. halopraeferens*. α -Ketoglutarate is used by *A. lipoferum* and by some strains of *A. irakense*. Sucrose has been used as the main carbon source for N_2 -dependent growth of *A. amazonense* and *A. irakense*. Studies using ^{14}C as a tracer have been carried out to evaluate the uptake of D-fructose, D-glucose, sucrose, mannitol, and α -ketoglutarate by *A. brasilense*, *A. lipoferum*, and *A. amazonense* (see Döbereiner and Pedrosa, 1987, for more details). The results confirmed the carbon metabolism already known for these species through use of other methodologies. No similar type of study has yet been carried out for the other *azospirilla* species.

Metabolic characteristics All enzyme activities of the catabolic Embden–Meyerhof–Parnas pathway, the Entner–Doudoroff pathway, and the tricarboxylic acid cycle have been detected in *A. brasilense*, *A. lipoferum*, and *A. amazonense*. However, there are differences among these species, as pointed out by Döbereiner and Pedrosa (1987) and Hartmann and Zimmer (1994) and reviewed here. Unfortunately, no similar studies have been carried out for the other species of *Azospirillum*. The type strains of *A. brasilense* and *A. lipoferum* have been found to phosphorylate fructose to fructose-1-phosphate by means of a phosphoenolpyruvate phosphotransferase system (Goebel and Krieg, 1984). Although both *A. brasilense* and *A. lipoferum* possess glucokinase, only *A. lipoferum* is permeable to D-glucose. Hexokinase activity occurs only in *A. lipoferum*. Sucrose-grown *A. amazonense* cells catabolize D-glucose and D-fructose (produced by the action of β -fructofuranosidase on sucrose) exclusively by the Entner–Doudoroff pathway (Martinez-Drets et al., 1985). *A. amazonense* strains possess fructokinase and glucokinase but not hexokinase. Enzymes of the Embden–Meyerhof–Parnas pathway and the Entner–Doudoroff pathway occur in D-fructose-grown cells of *A. brasilense* and *A. lipoferum*. High levels of NAD(P)-glucose 6-P-dehydrogenase, required for 6-phosphogluconate synthesis, have been detected in strains of *A. lipoferum* and *A. amazonense* but not in *A. brasilense* (Martinez-Drets et al., 1985). On the other hand, the enzyme 1-phosphofructokinase, involved in the phosphorylation of D-fructose, is present in large amounts in *A. lipoferum* and *A. brasilense* but absent in sucrose-grown *A. amazonense*. All three species above lack the key enzyme of the oxidative branch of the hexose monophosphate pathway (HMP). D-Gluconate is metabolized via the Entner–Doudoroff pathway in *A. lipoferum* and *A. brasilense*. Although *A. amazonense* possesses all three enzymes involved in the metabolism of D-gluconate it is unable to grow on this carbon source, probably because it lacks a gluconate transport system (Martinez-Drets et al., 1985). No information is yet available for the other species.

A. lipoferum exhibits weak fermentative ability in media containing D-glucose or D-fructose as a carbon source and with a source of fixed nitrogen. *A. largimobile* possesses fermentative ability in media containing carbohydrate, as determined by the Hugh–Leifson test (Ben Dekhil et al., 1997a). Although strains from all *Azospirillum* species grow far better aerobically than an-

aerobically, strains of *A. largimobile* have been considered facultative anaerobes. *A. lipoferum* is capable of acidifying D-glucose or D-fructose media anaerobically, of forming very small amounts of gas in Durham vials, of exhibiting slight growth in D-glucose or D-fructose broth under anaerobic conditions, and of forming minute colonies on D-glucose or D-fructose agar anaerobically. Variable results for these tests occur within some strains, and variants with decreased fermentative ability can be selected. Such variants continue to require biotin, use D-glucose as a sole carbon source for N₂-dependent growth, and exhibit the characteristic pleomorphic changes associated with this species. *A. largimobile* also produces acid (but no gas) from D-fructose, D-glucose, D-galactose, D-ribose, and sucrose, whereas *A. halopraeferens* produces acid from D-fructose but only aerobically. No acid is produced from D-glucose or D-fructose in *A. brasilense*, *A. amazonense*, and *A. irakense* either aerobically or anaerobically in a peptone-based medium. Acid is also produced from D-fructose and D-glucose in *A. doebereineriae* strains grown anaerobically (API50 test) (Eckert et al., 2001).

By an auxanographic method using media containing ammonium sulfate as the nitrogen source (Tarrand et al., 1978), the following compounds serve universally as sole carbon sources for all *A. lipoferum*, *A. brasilense*, and *A. halopraeferens* strains: malate, succinate, lactate, pyruvate, fumarate, β -hydroxybutyrate, D-gluconate, glycerol, and D-fructose. *A. irakense* and *A. amazonense* are also able to use these organic acids as carbon source; however, *A. amazonense* strains require a pH of approximately 6.0 in order to use the organic acids for N₂-dependent growth. No data are available for the use of organic acids as carbon source in *A. largimobile*. D-Gluconate and glycerol are not used by *A. amazonense* and *A. irakense*, whereas *A. largimobile* uses glycerol but not D-gluconate. D-Fructose is also catabolized by the other azospirilla species although variable results have been observed for *A. irakense*. Citrate and D-mannitol are not used by strains of *A. amazonense*, *A. irakense*, and *A. brasilense*. A few strains of *A. halopraeferens* do not catabolize citrate. D-Glucose is used by all azospirilla species except *A. halopraeferens* and most strains of *A. brasilense*. However, *A. brasilense* cannot use D-glucose as a sole carbon source for N₂-dependent growth, and it also produces a lower degree of acidification of glucose than does *A. lipoferum* (Tarrand et al., 1978). The use of D-galactose as carbon source is variable among strains of *A. brasilense* and negative for all strains of *A. halopraeferens*, whereas sucrose is catabolized only by strains of *A. amazonense* and *A. irakense*. N-Acetylglucosamine is used by *A. largimobile*, *A. lipoferum*, *A. irakense*, variable for *A. amazonense* strains but not used by *A. brasilense* and *A. doebereineriae*. No information is available for *A. halopraeferens*.

The following tests are universally positive for the genus *Azospirillum*: oxidase, phosphatase (weak in *A. largimobile*), urease, and esculin hydrolysis. The following tests are universally negative: starch and gelatin hydrolysis, production of water-soluble pigments, and indole production. Acidification of media containing lactose, sucrose, L-rhamnose, cellobiose, erythritol, dulcitol, or melibiose differs among strains and species of *Azospirillum*. Catalase activity ranges from strong to undetectable. The ability to grow anaerobically with nitrate in peptone-based media is positive for *A. lipoferum*, *A. brasilense*, and *A. halopraeferens* but negative for *A. amazonense*, *A. irakense*, and *A. largimobile*. In addition, the ability to dissimilate nitrate to either nitrite or to nitrous oxide and nitrogen gas differs among strains and species of *Azospirillum*. Most strains of *A. lipoferum*, *A. brasilense*, *A. halopraeferens*, and *A. doebereineriae* are able to denitrify. No denitrification has been observed in strains of *A. amazonense*, *A. irakense*,

and *A. largimobile*, although a report has been published showing a very low rate of denitrification for strain Y1 of *A. amazonense* (Neuer et al., 1985). Hybridization of DNA from five azospirilla species against the *nozZ*-segments containing the nitrous oxide reductase gene from *P. stutzeri* as a probe produced a positive signal with strains of *A. lipoferum*, *A. brasilense*, and *A. halopraeferens* but not for *A. amazonense* and *A. irakense*, confirming the presence of a N₂O reductase in the former three species and its absence in the last two species (Zimmer et al., 1995).

Pectinolytic activity (pectate lyase and pectin methylesterase) has been detected in strains of *A. irakense* (Khammas and Kaiser, 1991). Strains from this species are able to grow and fix nitrogen when pectin is used as the sole carbon source. Pectolytic activity—pectic lyase and endogalacturonase (Umali-Garcia et al., 1980) and polygalacturonic acid transeliminase (Tien et al. 1981)—has also been reported for some strains of *A. lipoferum* and *A. brasilense*. However, the enzymatic activity appears to be weaker than that of *Erwinia* (Tien et al., 1981). In addition, Khammas and Kaiser (1991) failed to demonstrate pectinolytic activity in these two species of *Azospirillum* as well as in strains of *A. amazonense*. Quite recently it was observed that *A. irakense* strain KBC1 also possesses cellulolytic activity—endoglucanase, cellobiohydrolase, and β -glucosidase activities (Vande Broek et al., 1998). No similar activities (pectinolytic and cellulolytic) have been tested for strains of *A. halopraeferens*, *A. largimobile*, and *A. doebereineriae*.

Production of the plant growth substance indoleacetic acid, and also indolelactic acid, gibberellin, and cytokinin-like substances, has been reported in strains of *A. brasilense* (Reynders and Vlassak, 1979; Tien et al., 1979). The production of indole-3-acetic acid by *A. brasilense* and *A. lipoferum* strains has been confirmed by many authors (Hartmann et al., 1983; Fallik et al., 1989; Bar and Okon, 1995) and the pathway for synthesis of IAA as well as the regulatory mechanism involved have been suggested (see Hartmann and Zimmer, 1994; Vande Broek et al., 1998). However, the production of cytokinins and gibberellins is still not clear because these products were detected only via bioassays in very old cultures (see Hartmann and Zimmer, 1994). More recently, the production of abscisic acid in *A. brasilense* (Iosipenko and Ignatov, 1995) and gibberellic acid in strains of *Azospirillum lipoferum* (Bottini et al., 1989) and other azospirilla (Rasul et al., 1998) was demonstrated using the HPLC technique. However, the production was detected with 10-d-old cells. The production of IAA in *Azospirillum irakense* is very low and the gene *trpD*, encoding phosphoribosyl anthranilate transferase, was proposed to be involved in the repression of IAA production (Zimmer et al., 1991). No information about the production of phytohormones by the other *Azospirillum* species is available. Plant response to inoculation with *Azospirillum*, mainly increase in root area, has been reported and attributed to phytohormones such as IAA produced by these bacteria (Bashan and Holguin, 1997).

Other substances such as the vitamins thiamin, niacin, pantothenic acid (Rodelas et al., 1993) and riboflavin (Dahm et al., 1993) have also been detected in *A. brasilense* strains. It has also been reported that *Azospirillum lipoferum* is able to degrade 4-chloronitrobenzene (Russel and Muszynski, 1995) and more recently it was demonstrated that many strains (73 out of 110) of *A. lipoferum* and *A. brasilense* are able to produce cyanide (Gonçalves and Oliveira, 1998). The ability to oxidize phenolic derivatives like syringic aldehyde, acid, or acetosyringone has been detected in *A. lipoferum* strain 4T and is carried out by laccase—an oxidase widespread in fungi and higher plants (Faure et al., 1996).

Azospirillum spp. isolated from saline soils exhibited different

degree of osmotolerance. *A. halopraeferens*, isolated from sodic alkaline soils of Pakistan, exhibited the highest osmotolerance, followed by *A. brasilense*, *A. lipoferum*, and *A. amazonense* (Hartmann, 1988); the osmotolerance of *A. irakense* lies between *A. halopraeferens* and *A. brasilense* (Khammas et al., 1989). As compatible solutes in *Azospirillum*, trehalose, glycine betaine, glutamate, and proline were characterized (Hartmann et al., 1991). *A. halopraeferens* is able to take up choline efficiently and to convert it into the most potent osmoprotectant, glycine betaine (Hartmann, 1988; Hartmann et al., 1991). In *A. brasilense* Sp7, a binding protein-dependent, high-affinity uptake of glycine betaine was demonstrated and glycine betaine stimulated growth and nitrogen fixation in *A. brasilense* (Hartmann, 1988; Riou and Rudulier, 1990). In osmotolerant *Azospirillum* spp., choline and glycine betaine, as well as glutamate, proline, and other amino acids, are not or only slightly used as nitrogen or nitrogen and carbon sources (Hartmann et al. 1988; Khammas et al., 1989). Dehydropyrolone (DHP)-resistant bacteria with improved osmoregulatory properties are available for *A. brasilense* Sp7 (Hartmann et al., 1992). The fact that these mutants appear spontaneously at relatively high rates suggests the ecophysiological importance of this trait.

Under iron-limiting conditions, *A. lipoferum* and *A. brasilense* release the phenolate siderophores 2,3- and 3,5-dihydroxybenzoic acid conjugated with lysine and leucine or ornithine and serine, respectively, which has been called spirillobactin (Saxena et al., 1986; Bachhawat and Ghosh, 1987b). The Fe(III)-spirillobactin complex is taken up via a high-affinity uptake receptor, which involves a specific outer membrane receptor (Bachhawat and Ghosh, 1987a). It has been shown for *A. brasilense* Sp245 that the siderophores ferrichrome, ferrichrysin, and coprogen, as well as the main siderophore of *Streptomyces*, ferrioxamine B, can be used as iron sources too (Hartmann, 1988). However, the iron-scavenging properties vary much among different *Azospirillum* species and strains; e.g., *A. brasilense* strain Sp7 was only a weak siderophore scavenger (Hartmann, 1988). Under severe iron-limiting conditions, which drastically inhibit the growth of *A. brasilense* Sp7 wild type, spontaneous mutants that could readily use coprogen- and ferrichrysin-bound iron, appeared (Hartmann et al., 1992). The high-affinity iron uptake systems of *A. halopraeferens*, *A. irakense*, and *A. doebereinae* have not yet been studied. Among azospirilla, only *A. irakense* isolates can efficiently hydrolyze desferrioxamine (Winkelmann et al., 1999). The ecological relevance of this unique physiological property has not yet been elucidated. High-affinity-mediated siderophore acquisition could be an important trait for competitiveness in a highly populated habitat such as the rhizosphere.

Genetic characteristics Genetic transformation to generate antibiotic-resistant derivatives has been reported for *A. brasilense* ATCC 29145 (Mishra et al., 1979). Genetic transformation has been also demonstrated for other strains of *A. brasilense* and *A. lipoferum* (Wood et al., 1982; Fani et al., 1986). More recently, DNA transformation of *Azospirillum* using a high-voltage electroporation mechanism was developed (Vande Broek et al., 1989); however, the methodology worked only for *A. brasilense* strains and not for *A. lipoferum* strains. There are no reports of DNA transformation for the other *Azospirillum* species. In contrast to transformation, conjugation appears to be the most efficient method of gene transfer for *Azospirillum* species (see Döbereiner and Pedrosa, 1987). More recently, a *nifH*-*gusA* fusion plasmid pFAJ21 was transferred by a biparental mating to *A. irakense* (Vande Broek et al., 1996). However, there are no reports of conjugation for the other described *Azospirillum* species.

Genetic recombination has been demonstrated in *A. brasilense* ATCC 29145, using plasmid R68-45 (derived from *Pseudomonas aeruginosa*) to mobilize the chromosome (Franché et al., 1981). The mode of gene transfer promoted by the plasmid appeared to be unpolarized, suggesting the existence of multiple origins of transfer. Similar results were obtained by Bazzicalupo and Galiori (1983) using strains Sp7 and Sp6 of *A. brasilense*. Although much progress has been made, the data obtained so far do not allow a genetic map to be drawn for the chromosome of *A. brasilense*. Several difficulties in constructing the genetic map using this technique have been pointed out by Döbereiner and Pedrosa (1987). Nevertheless, most of the genes involved in the nitrogen fixation process have already been identified in *A. brasilense* and *A. lipoferum* and located on the chromosome (Vande Broek and Vanderleyden, 1995). In addition, the organization and regulation of the *nif* genes as well as the genes involved in nitrogen metabolism are well established in *A. brasilense* (Elmerich et al., 1997). Little information is available so far for the other *Azospirillum* species.

Plasmids and bacteriophages The occurrence of multiple large plasmids in *A. brasilense* and *A. lipoferum* strains was reported by Wood et al. (1982). The authors called these megaplasmids "minichromosomes" that varied in size from 42 to 1850 MDa. Two plasmids of very high molecular weight (1700 and >1800 Kb) have also been observed in several strains (24 out of 28) of *A. brasilense* isolated from sugarcane plants (Caballero-Mellado et al., 1999). These authors also observed that the megaplasmid of 1700 Kb and the plasmid of approximately 600 Kb carry genes homologous to the 16S rDNA, thus reinforcing the hypothesis of the presence of minichromosomes in *Azospirillum brasilense*. More recently, the genome structure of five *Azospirillum* species was analyzed by pulsed-field gel electrophoresis (Martin-Didonet et al., 2000). The authors detected the presence of 8–10 replicons in the species *A. lipoferum* and *A. brasilense* (0.15–2.5 Mbp) and 4–5 in the species *A. amazonense*, *A. irakense*, and *A. halopraeferens* (0.22–2.7 Mbp). They also observed the hybridization of a 16S rDNA probe to some replicons, confirming the existence of multiple chromosomes in the genus. In addition, the authors observed that the *nifHDK* operon is present in the largest replicon. Strains of *A. brasilense* and *A. lipoferum* harboring 1–7 plasmids ranging from 4 to 310 MDa have been described by other authors (Franché and Elmerich, 1981; Plazinski et al., 1983). Plasmids have also been detected in strains of *A. amazonense* and *A. halopraeferens*; however they are present in low numbers and are small in size (Elmerich et al., 1991). No information is yet available for *A. doebereinae*. Many reports have shown that *Azospirillum* plasmids harbor genes involved in the plant–bacterium interaction and other physiological functions. A plasmid called p90, present in many *A. lipoferum* and *A. brasilense* strains, carries genes involved in chemotaxis, motility, and plant root adsorption (Michiels et al., 1994; Vande Broek and Vanderleyden, 1995). In addition, it also carries *nod* and *exo* genes that are homologous to those frequently found in legume-nodulating rhizobia (Onyeocha et al., 1990). Other functions such as production of melanin in *A. lipoferum* (Givaudan et al., 1991) and IAA biosynthesis in *A. brasilense* (Katzy et al., 1995) have also been detected. The p90 plasmid tagged with Tn5 was mobilized to *Agrobacterium tumefaciens* at a frequency of 10^{-4} , but self-transfer of p90 was not demonstrated (Onyeocha et al., 1990).

Lysogenicity is common in *Azospirillum* (Döbereiner and Pedrosa, 1987). It has been detected in many strains of *A. lipoferum* and *A. brasilense* and the lysis is inducible by mitomycin C (Fran-

che and Elmerich, 1981; Elmerich et al., 1982). However, not all strains of these two species are lysogenic. Temperate bacteriophage Al-1—isolated from soil samples from Brazil—was purified and showed a size and morphology similar to that of coliphage λ (Elmerich, 1983). This phage is lysogenic to some strains of both species (see Döbereiner and Pedrosa, 1987). Phage Ab-1 particles from *A. brasilense* Sp7 were characterized and shown to be infective to strains Sp7 and Cd of *A. brasilense* but not to strains Sp35, Sp59a, RG20a, and Br17 of *A. lipoferum* (Germida, 1984). The presence of bacteriophages in strains of the other species of *Azospirillum* has not been determined.

Bacteriocins Production of bacteriocins has been observed in many strains of *A. lipoferum* and *A. brasilense* in pure culture, but this seems not to be the case for strains living in soil or associated with roots (Oliveira and Drozdowicz, 1981; 1988). Catechol-type siderophores with antimicrobial activity against various bacterial and fungal isolates have been detected in 27 strains of *Azospirillum* (Shah et al., 1992).

Antigenic characteristics Antisera prepared against whole cells have been used in the indirect fluorescent antibody technique to distinguish between *A. lipoferum* and *A. brasilense* and also between groups of strains (Schank et al., 1979; De Polli et al., 1980). Several other reports applying polyclonal antisera to evaluate the colonization of grass plants by *A. lipoferum* and *A. brasilense* have been published (see Kirchhof et al., 1997b, for more details). More recently, with the help of hybridoma technology, several strain-specific monoclonal antibodies were developed for *A. brasilense* (Kirchhof et al., 1997b). Three classes of monoclonal antibodies with high specificity for *Azospirillum brasilense* Sp7 have been characterized by Schlöter et al. (1994). One class recognizes a 100 kDa protein subunit of the polar flagellum, and the other two bind to an 85 kDa outer membrane protein and to polysaccharide, respectively. Monoclonal antibodies specific for *A. brasilense* strain Sp245 and *A. brasilense* strain Wa5 were recently characterized (Schlöter and Hartmann, 1996; Schlöter et al., 1997). As antibody-binding cell surface components, lipopolysaccharides and outer membrane proteins were identified. Studies of wheat root colonization by the above strains using the strain-specific monoclonal antibodies showed that strain Sp245 colonizes the inner root tissue endophytically whereas strain Sp7 colonizes only the root surface (Schlöter and Hartmann, 1998). No monoclonal antibodies for other *Azospirillum* species have been produced; therefore, strain-specific root colonization studies involving other species are lacking.

Ecology *Azospirilla* have a worldwide distribution and occur in large numbers (up to 10^7 /g) in rhizosphere soils and in association with the roots, stems and leaves of a large variety of different plants. Most isolates have been obtained from tropical forage grasses and from cereal plants grown in tropical regions. However, *azospirilla* have also been frequently isolated from plants grown in temperate regions, including graminoids grown in the Canadian High Arctic sites (Nosko et al., 1994). The idea that *Azospirillum* species were limited to grass plants is no longer tenable. *A. lipoferum* and *A. brasilense* have a widespread occurrence and colonize various plants including legumes, vegetables, and fruits (Bashan and Holguin, 1997; Kirchhof et al., 1997a; Weber et al., 1999). *A. amazonense* has been isolated from roots of maize, sorghum, rice, wheat, forage grasses, and palm trees grown around Brazil. It has also been isolated from roots and stems of sugarcane plants grown in Brazil, from sugarcane roots grown in Hawaii and Thailand, and more recently, from pine-

apple and banana fruits grown in Brazil (Weber et al., 1999). The other *Azospirillum* species appeared to be more restricted: *A. halopraeferens* has so far only been found associated with Kallar grass plants grown in Pakistan. *A. irakense* has so far been isolated from rice plants grown in Iraq (Khammas et al., 1989) and from a freshwater pond in the botanical garden in Tübingen, Germany (Winkelmann et al., 1996). *A. largimobile* has only been isolated from a freshwater lake located in Australia (Ben Dekhil et al., 1997a) while *A. doebereineriae* was so far isolated from roots of the C₄-graminaceous plant *Miscanthus* grown in Germany (Eckert et al., 2001).

In field-grown maize, *azospirilla* occur on the surface of roots, in the outer cortex, inner cortex, and in the stele (Patriquin and Döbereiner, 1978). Infection of the inner cortex and stele occurs in the absence of significant bacterial colonization or collapse of outlying tissues. Paraxylem vessels can be completely plugged with the bacteria. Infection occurs initially in root branches and spreads longitudinally into main roots. In monoaxenic cultures of pearl millet and guinea grass, *azospirilla* are found within the mucigel layer of roots and become firmly attached to root hairs. The bacteria enter the roots through lysed root hairs and void spaces created by epithelial desquamation and lateral root emergence (Umali-Garcia et al., 1980). *Azospirilla* have been observed in intercellular locations within the middle lamella of root tissues; they have also been observed intracellularly, sometimes in very large numbers.

The above observations of root colonization were recently confirmed for *A. brasilense* strains Sp245 and Sp7 inoculated into wheat plants and evaluated using sophisticated techniques such as confocal laser scanning microscopy (SCLM) coupled with fluorescent probes (Assmus et al., 1995), as well as the use of strain-specific monoclonal antibodies (Schlöter and Hartmann, 1998) and *nifH*-*gus* fusion (Vande Broek et al., 1993). There was a marked predominance of *A. brasilense* in the root hair zone as compared with the root tips of wheat plants. *A. brasilense* strain Sp245 was repeatedly detected in the interior of root hairs whereas *A. brasilense* strains Sp7 and Wa3—both isolated from a rhizosphere—colonized only the rhizoplane (Assmus et al., 1995). Strain Sp245, already known to be a colonizer of the root interior (Baldani et al., 1986b), could be found in the intercellular space of the root parenchyma and inside cortex and parenchyma cells, with the point of emergence of lateral roots being the most probable infection site (Assmus et al., 1997). More recently, it was observed that microcolonies are formed in the intercellular space of wheat roots inoculated with strain Sp245, but not with strains Sp7 and Wa3, and that the plant genotype influenced root colonization (Schlöter and Hartmann, 1998). Use of strain Sp245 expressing the *gusA* gene showed that the bacteria initially concentrate in the root-hair zones and at sites of lateral root emergence. Proliferation to the other parts of the root was dependent on the status of the nitrogen and carbon sources present in solution (Vande Broek et al., 1993). Although the complete process of association between *Azospirillum* and the host grass plant is still unclear and a model has already been suggested (Del Gallo and Fendrik, 1994), it is known that the colonization requires the attachment of the cell to the root surface. This attachment occurs in two steps: one that is fast, weak, and probably mediated by bacterial proteins, and the second phase (also called anchoring) which is longer and irreversible and probably involves extracellular surface polysaccharides and the polar flagellum (see Vande Broek and Vanderleyden, 1995, for more details). It has been suggested that the *rpoN* gene, involved in nitrate reduction, nitrogen fixation, and cell motility,

also controls the colonization of wheat plants by *A. brasilense* Sp7 (Pereg-Gerk et al., 1998). Because the ability of most strains of *Azospirillum* to colonize both the surface and root interior, including the stems and leaves of grass, these bacteria were included in the group called facultative endophytic diazotrophs (Baldani et al., 1997). This term was suggested to distinguish them from the obligate endophytic bacteria that include *Gluconacetobacter diazotrophicus*, a bacterium found colonizing sugarcane plants endophytically (Döbereiner, 1992b).

Several field inoculation experiments applying mainly strains of *A. brasilense* and *A. lipoferum*, and in a few cases *A. amazonense*, have been carried out in the last 20 years at various locations around the world. About 60–70% of all field experiments showed positive effects of the inoculation, with significant yield increases ranging from 5 to 30% (Okon and Labandera-Gonzalez, 1994; Baldani et al., 1997). Few commercial inoculants are available, probably because of the inconsistency and low percentage of response to inoculation. There is still a debate about the main mode of action by which these bacteria contribute to the nitrogen accumulated by the plants. Effects of plant-growth promoting substances, nitrogen fixation *per se*, and the ability of the bacterial nitrate reductase to help in the incorporation of nitrogen assimilated from the soil by the plant (Baldani et al., 1997) have also been mentioned as the major factors involved in the response of the plant to *Azospirillum* inoculation.

ENRICHMENT AND ISOLATION PROCEDURES

Tenfold serial dilutions of roots, stems, or leaf samples are inoculated into 10-ml cotton-plugged serum vials containing 5 ml of semisolid medium and incubated for up to one week at the optimal temperature. Soil samples or smashed pieces (5–8 mm long) of plant tissues can also be used but the incubation time should be only 40–48 h. Nitrogenase activity can be tested by acetylene reduction assay for those vials containing pellicles on the surface.

For vials exhibiting acetylene reduction, a second serial transfer is made. In the case of *A. brasilense*, *A. lipoferum*, and *A. doebereineriae*, the pellicle is transferred to semisolid NFb medium. After 24 h, the growth is streaked out on plates of solid NFb medium containing 0.02 g/l yeast extract. After 1 week, typical small, white, dense, single colonies are sub-cultured to semisolid NFb medium, where subsurface growth in the form of a veil is a presumptive indication of successful enrichment. For final purification, cultures are streaked onto BMS agar and the typical pink, often wrinkled, colonies are transferred for storage and identification (Döbereiner et al., 1995).

For *A. amazonense*, the pellicle is transferred to semi-solid LGI medium⁵ and after 3–4 d streaked out on plates of LGI medium containing 0.02% yeast extract. After 5 d, small (2 mm diameter) whitish colonies with firm, dense, but not tenacious consistency and which are partially imbedded into the agar are selected and transferred to new LGI vials and, if growing well, streaked on BMS agar for final purification. On this medium, large, white, flat colonies with raised margin are formed.

5. LGI medium (g/l): Sucrose, 5.0; K₂HPO₄, 0.2; KH₂PO₄, 0.6; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.02; Na₂MoO₄·2H₂O, 0.002; bromothymol blue 0.5% in 0.2 N KOH, 5ml; FeEDTA (1.64% solution), 4 ml; vitamin solution (biotin, 0.01g; pyridoxal-HCl, 0.02g; distilled water, 100ml), 1 ml; pH adjusted to 6.0–6.2 with H₂SO₄ and the final volume completed to 1000 ml with distilled water. For semi-solid medium, 1.8 g/l agar was added. For solid medium, add 15 g/l agar and 20–50 mg/l yeast extract.

For *A. halopraeferens*, roots of *Leptochloa fusca* plants are collected and the rhizoplane soil separated, diluted, and inoculated into semi-solid modified NFb medium, named SM⁶ (containing vitamins, 0.25% NaCl; pH adjusted to 8.5), and incubated at 41°C. Culture from vials showing nitrogenase activity, as determined by the acetylene reduction method, are then transferred to fresh semi-solid SM medium and after 48–72 h of incubation the pellicle is suspended in the same medium containing yeast extract (0.02%). Aliquots are then used to seed molten, cooled (45°C), soft SM (0.8% agar), which is then poured into Petri dishes and incubated for 1 week. Small, white colonies are visible within the agar and are further purified by embedding in the same way. For final purification, colonies are again transferred to semi-solid SM medium and streaked on Tryptic soy agar; cream-colored, circular, flat colonies with an entire margin are formed. No growth occurs on BMS or Congo red agar medium.

For *A. irakense* species, rhizosphere and roots of rice plants are crushed, suspended in sterile distilled water, diluted, and inoculated into semisolid NFb medium. The intermediate purification steps basically follow those for *A. lipoferum*. The final purification is performed on modified semisolid AAM medium⁷ (containing 4 ml/l of a 1.64% solution of FeEDTA; pH of medium adjusted to 6.5). Subsurface pellicles are streaked out on the modified AAM agar plates. Colonies formed after 4 d are translucent, glistening, and convex with a regular margin, 1 mm diameter.

In the case of *A. largimobile*, one drop of fresh lake water is spread onto the surface of lake water agar (LWA) plates, dried and incubated for up to 8 d at 28°C. Colonies grown on LWA for 72 h at 28°C are 1–2 mm in diameter, colorless, translucent, low convex, and round, with an entire edge and smooth surface and they readily coalesce. These colonies, containing unicellular or multicellular forms, are further purified on LWA agar where very active motile cells containing refractile granules appear 4–8 h after incubation. These motile cells have a striking resemblance to cells of *Beijerinckia* species. To ensure that the phenomenon of multicellular form occurs, the cells are again inoculated in LWA or lake water salt agar (LWSA). Although *A. largimobile* has shown these peculiar characteristics, strains from this species are able to grow and fix nitrogen in semisolid NFb medium, with many aspects of their growth resembling *A. lipoferum*.

The following method has been used for the specific enrichment of *A. lipoferum*. A nitrogen-free mineral medium is inoculated with soil samples or washed root pieces and incubated under a gas atmosphere of O₂/CO₂/H₂/N₂ (5:10:30:55). After one

6. SM medium (g/l): DL-malic acid, 5.0; KOH, 4.8; NaCl, 1.2; NaSO₄, 2.4; NaHCO₃, 0.5; CaCl₂, 0.22; MgSO₄·7H₂O, 0.25; K₂SO₄, 0.17; Na₂CO₃, 0.09; Fe-EDTA, 0.077; K₂HPO₄, 0.13; biotin, 0.0001; MnCl₂·4H₂O, 0.0002; H₃BO₃, 0.0002; ZnCl₂, 0.00015; CuCl₂·2H₂O, 0.000002; Na₂MoO₄·2H₂O, 0.002; distilled water, completed to 1000 ml. The final pH of the medium is 8.5 and it does not require further adjustment. For semisolid medium, 2.0 g/l agar were added. Malic acid, KOH, and agar are dissolved in one-half of the total volume and autoclaved. The remaining salts are sterilized by filtration after dissolving them in one-half of the total volume and discarding the precipitate after centrifugation. For solid medium, 8 g/l agar and 20 mg/l yeast extract were added.

7. AAM defined medium (g/l): K₂HPO₄, 2.0; KH₂PO₄, 6.0; MgSO₄·7H₂O, 0.2; NaCl, 0.1; CaCl₂·2H₂O, 0.026; Na₂MoO₄·2H₂O, 0.002; FeCl₃, 0.01; NH₄Cl, 1.0; distilled water, 1000 ml. The pH is adjusted to 6.0. For semisolid medium, 1.9 g/l agar were added and the NH₄Cl was omitted. For solid medium, 15 g/l agar were added. The carbon sources are sterilized separately (i.e., DL-sodium malate) or filter sterilized (i.e., sucrose) and added aseptically to the minimal medium to give a final concentration of 0.03M.

week, 0.5 ml of the suspension is transferred to fresh medium and incubated as before. The procedure is repeated 2 or 3 times before serial dilutions are plated onto nitrogen-free mineral media containing 1.5% (w/v) agar. Colonies are selected and propagated under autotrophic conditions.

A method has been developed and used to isolate *A. lipoferum* from the rhizosphere of rice plants (Tran Van et al., 1997). Serial dilutions of crushed roots and rhizosphere soil suspensions are inoculated into 18 × 180-mm tubes, each tube containing 8 ml of one of four different liquid N-free media: distilled water, KCl solution (8.5 g/l), NFB liquid medium, and soil extract (obtained by autoclaving a soil suspension in water (50% w/v) at 130°C for 1 h and filtering through cellulose filters followed by autoclave treatment). The tubes are incubated at 30–32°C for 15 d in the dark to prevent growth of algae or cyanobacteria. A pellicle is formed 2 mm below the surface of the liquid, and when examined under the microscope normally shows the presence of spiral motile cells. Samples (10 µl) of the pellicle, from the highest dilution, are plated on nutrient agar medium and incubated for 48–72 h. All colony types are then purified. The authors claim that these methods are much simpler than the use of semisolid media; however, many more colonies have to be checked for the presence of nitrogen-fixing ability.

MAINTENANCE PROCEDURES

Stock cultures of *A. brasilense*, *A. lipoferum*, and *A. doebereineriae* may be maintained in semisolid NFB medium at 8–30°C with monthly transfers for *A. brasilense* and biweekly transfers for *A. lipoferum* (Döbereiner, 1992a). Stocks may also be maintained on trypticase soy agar with monthly transfer (Tyler et al., 1979). *A. brasilense* remains viable for several years and *A. lipoferum* for 3–6 months in sterile vermiculite moistened with potato broth; drying should be avoided by tightly sealing the vials with screw caps. A combination of soil and farmyard manure (1:1) can maintain a population of *A. brasilense* at high levels for up to 6 months (Tilak et al., 1979).

Strains can also be kept lyophilized for many years. Cells grown on slanted NFB medium (malic acid is replaced by glucose for *Azospirillum lipoferum* strains) for 48–72 h at 30°C are suspended in 2 ml of a lyophilization solution consisting of 10% sucrose and 5% peptone in 100 ml water. Aliquots (0.2 ml) are then distributed into lyophilization ampules and lyophilization performed as recommended for rhizobia.

For preservation in liquid N₂, heavy suspensions of cells harvested from MPSS broth or MPSS agar plates are prepared in nutrient broth containing 10% (v/v) dimethyl sulfoxide (Tarand et al., 1978) or in TSS broth (1% trypticase, 0.5% succinate with salts as in NFB medium) in which 15% of the water is replaced by glycerol (Tyler et al., 1979). The suspensions are placed in vials and preserved in liquid nitrogen.

A. amazonense strains may also be maintained in semisolid LGI medium at 30°C with biweekly transfers. Strains from this species have been maintained for more than 15 years in test tubes containing LGI agar medium supplemented with yeast extract and covered with mineral oil. The cultures should never be stored in BMS agar medium even under oil because of the pH sensitivity of the bacteria. Cultures can also be maintained lyophilized after growth for 48–72 h at 30°C in a medium with sucrose as the sole carbon source and following the procedure described above.

A. halopraeferens cells may be maintained at 41°C by biweekly transfer in semi-solid SM medium supplemented with 0.25%

NaCl and adjusted to pH 7.2 (Reinhold et al., 1987). They can also be maintained in liquid nitrogen as described for the other species of *Azospirillum*. *A. irakense* cells are maintained at room temperature on slants of modified AAM agar medium (Khammas et al., 1989) and *A. largimobile* can be maintained on LWA or LWSA agar medium according to the description by Skerman et al. (1983).

Azospirilla can also be maintained for long periods in water. Two colonies grown on the specific agar medium are transferred to micro tubes containing 0.5 ml of distilled sterile water and stored at room temperature. To revive, the cells are homogenized and 20–100 µl are transferred to semi-solid medium and incubated at the appropriate temperature.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

D-Glucose and sucrose used as sole carbon source for growth in semi-solid nitrogen-free medium For D-glucose, a loopful of culture from each species grown in the appropriate semi-solid or liquid medium is inoculated into a tube of semi-solid, nitrogen-free, D-glucose medium (the specific carbon source is replaced by 1.0% D-glucose which has been sterilized by filtration). *A. lipoferum*, *A. amazonense*, and most strains of *A. doebereineriae* form a veil or disc of growth in the depths of the medium; within 3 d at 35–37°C this disc migrates close to the surface of the medium and becomes very dense. *A. brasilense* and *A. halopraeferens* either give no response or form a slight pellicle in the depths of the medium that later disperses. The difference in response between these four species is very pronounced. The other two species, *A. irakense* and *A. largimobile*, also use D-glucose but this characteristic has not been checked under nitrogen-fixing conditions.

For sucrose, a loopful of culture from *A. amazonense* or *A. irakense* grown in liquid medium is inoculated into a vial of semi-solid LGI medium containing sucrose as sole carbon source, and the pH is adjusted to 6.0–6.2 for *A. amazonense* and 6.5 for *A. irakense*. *A. amazonense* forms a fine pellicle below the surface very similar to that of the other species in NFB medium except that growth is initially slower; however, a thick surface pellicle is formed after 3–4 d. *A. irakense* forms a white pellicle 2 mm below the surface after 1 d of incubation but reaches the surface within 2–3 d. The other species do not use sucrose as sole carbon source, although some variants of *Azospirillum lipoferum* have been found to use sucrose for growth.

Biotin requirement Glassware should be rinsed copiously with distilled water and subsequently baked in an oven to destroy traces of biotin. A medium of the following composition is used (g/l): K₂HPO₄, 0.5; succinic acid (free acid), 5.0; FeSO₄·7H₂O, 0.01; Na₂MoO₄·2H₂O, 0.002; MgSO₄·7H₂O, 0.2; NaCl, 0.1; CaCl₂·2H₂O, 0.026; (NH₄)₂SO₄, 1.0. The pH is adjusted to 7.0 with KOH solution. Biotin (0.0001 g/l) is added to one portion. The biotin-free and biotin-containing media are sterilized in 5.0-ml amounts in screw-capped tubes by autoclaving. Cultures grown in MPSS broth are inoculated by a loop into 25 ml of one-quarter-strength nutrient broth (Difco) and incubated at 37°C for 24 h. The cells are harvested by centrifugation, washed twice with 10-ml portions of sterile distilled water, and suspended in water to a turbidity of 20 Klett units (blue filter, 16-mm tubes). An aliquot (0.1 ml) of this suspension is used to inoculate each 5.0 ml of medium (with and without biotin). The cultures are incubated for 48 h at the optimal temperature for the species. In cases where growth occurs in the absence of biotin, a second serial transfer is made to media with and without biotin, using 0.1 ml

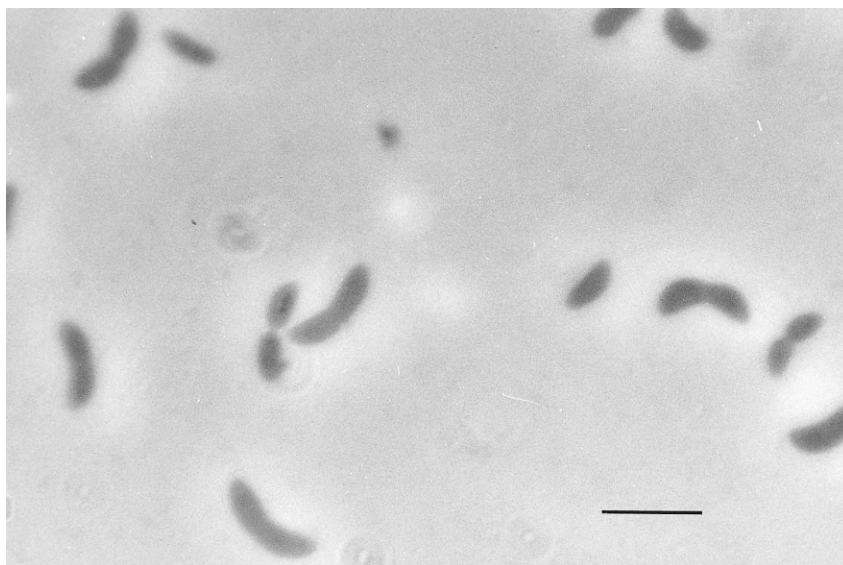


FIGURE BXII.α.10. *Azospirillum brasilense* ATCC 29145 cultured in MPSS broth at 37°C for 24 h. Phase-contrast microscopy. Bar = 5 μm.

from the first culture. This procedure may be repeated at least ten times to make sure that strains are not dependent on biotin.

Development of pleomorphic cells A loopful of growth from a 24 h-old MPSS broth culture is inoculated into a tube of semi-solid, nitrogen-free, malate medium (containing 0.05 g/l yeast extract or 0.0001 g/l biotin). The cultures are examined by phase-contrast microscopy after incubation at 37°C for 24–48 h (Fig. BXII.α.10). *A. brasilense* and *A. amazonense* remain mainly vibrioid and motile, whereas *A. lipoferum* becomes wider, longer, nonmotile, and S-shaped or helical. These forms of *A. lipoferum* eventually undergo fragmentation into shorter, ovoid cells, many of which become very large and pleomorphic and are filled with refractile granules. *A. halopraeferens* strains also develop wider, longer and S-shaped cells, which seldom exceed a length of 5 μm. In some cases, very long cells are also observed. Cells of *A. irakense* grown in complete nutrient broth medium are much longer and reach a size of up to 30 μm. *A. largimobile* cells lose motility and form multicellular conglomerates that become optically refractile and reproduce by multi-planar septation. *A. doebereineriae* strains remain motile after growth overnight in liquid medium, but the cells become long and pleomorphic after prolonged growth in semisolid N-free NFb medium.

Auxanographic method for sole carbon sources The following ingredients are dissolved in 50 ml of distilled water passed through a Bantam multibed resin cartridge (Barnstead Co., Boston, MA): (NH₄)₂SO₄, 1.0 g; MgSO₄·7H₂O, 1.0 g; K₂HPO₄, 2.0 g; FeCl₃·6H₂O, 0.0047 g; MnSO₄·H₂O, 0.0025 g; ZnSO₄·7H₂O, 0.00072 g; CuSO₄·5H₂O, 0.000125 g; CoSO₄·7H₂O, 0.00014 g; H₃BO₃, 0.000031 g; and Na₂MoO₄·2H₂O, 0.000245 g. The pH is adjusted to 2.5 with HCl to dissolve precipitates and then adjusted to 7.0 with KOH. Biotin (0.0001 g) and CaCO₃ (0.001 g) are added and the medium is sterilized by autoclaving. The sterile medium is added aseptically to an equal volume of sterile agar solution (15.0 g purified agar (Difco) in 500 ml of distilled water, sterilized by autoclaving) at 45–50°C. Cells are prepared as for the determination of biotin requirement (previous paragraph) except that the final suspension is adjusted to 30 Klett units

instead of 20. Two ml of the cell suspensions are used to spread 20 ml of molten medium at 45–50°C in a Petri dish. After solidification of the medium, sterile 7-mm diameter paper discs (punched from Beckman electrophoresis filter paper, Cat. No. 319328) are dipped into 5% (w/v) aqueous solutions of carbon sources sterilized by filtration. (Solutions of organic acids are adjusted to pH 7.0 with KOH before sterilization.) The saturated discs are then placed near the periphery of the seeded agar plates (3 discs/plate). The plates are incubated at 37°C for 72 h. Any visible zone of turbidity around the discs, as judged by holding the plates against a black background and illuminating them with oblique lighting from the rear, constitutes a positive growth response. New kits for testing these properties are now available on the market (i.e., Biolog, API galleries) and the instructions from the manufacturers should be followed.

Acidification of peptone-based D-glucose medium The following medium is used (g/l): peptone (Difco), 2.0; MgSO₄·7H₂O, 1.0; (NH₄)₂SO₄, 1.0; FeCl₃·6H₂O, 0.002; MnSO₄·H₂O, 0.002; bromothymol blue (dissolved in dilute KOH), 0.025. The medium is made up to a volume of 950 ml, adjusted to pH 7.0 and sterilized by autoclaving. After it has cooled, 50 ml of a 20% (w/v) solution of D-glucose (sterilized by filtration) is added aseptically. The development of a yellow color during incubation for 96 h at 37°C indicates acidification.

Fermentative ability Tubes of peptone–D-glucose or D-fructose media, or defined D-glucose or D-fructose broth (Tarrand et al., 1978), are inoculated and placed in GasPak jars (BBL) containing a fresh catalyst. The jars are evacuated, filled once with N₂ and three times with H₂, sealed and incubated at 37°C for up to 2 weeks. Development of a yellow color indicates acidification. Slight growth in the form of a sediment can be detected by agitating the tubes.

Test for autotrophic growth (Málek and Schlegel, 1981) A mineral medium having the following composition (per liter) is used: KH₂PO₄, 2.3 g; Na₂HPO₄·2H₂O, 2.9 g; NH₄Cl, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.01 g; ferric ammonium cit-

rate, 0.05 g; and trace element solution⁸, 6.0 ml. When necessary, the medium is supplemented with the appropriate growth factors. For testing in liquid culture, 20-ml portions of the medium are distributed into 100- or 250-ml Erlenmeyer flasks. After inoculation, the flasks are placed in desiccator jars or anaerobic jars. The jars are then evacuated and filled with the following gas mixture: O₂/CO₂/H₂/N₂ (2:10:60:28). The jars are incubated at 30–37°C with shaking and are periodically evacuated and re-filled with fresh gas mixture. For testing growth on agar plates, the mineral medium is solidified with 1.5% agar.

Oligonucleotide probes Probes complementary to a highly variable stretch of helix 55–59 of the 23S rRNA were designed and are available for five species of *Azospirillum* (Kirchhof et al., 1997b). Oligonucleotide probes based on partial 16S rRNA gene sequences have also been designed by Kabir et al. (1995) and were species specific for *A. lipoferum* and *A. amazonense*; another probe recognized both *A. brasilense* and *A. amazonense*. The sequences and dissociation temperatures (°C) of probes used for phylogenetic studies of azospirilla are shown in Table BXII.α.3. Bulk nucleic acids are isolated from strains that were cultivated overnight in nutrient broth or DYGS liquid medium (g/l: dextrose, 2.0; peptone, 1.5; yeast extract, 2.0; K₂HPO₄, 0.5; MgSO₄, 0.5; L-glutamic acid 1.5; pH adjusted to 6–6.5), and transferred to a positively charged nylon membrane via spot blotting. Hybridization with radioactive or nonradioactive DIG-labeled probes is performed for 2–12 h (radioactive) or 16 h (nonradioactive) according to the method of Kirchhof et al. (1997a). Signals are detected by autoradiography. Most recently, a comprehensive set of whole-cell-binding 16S rRNA-targeted oligonucleotide probes was developed at genus and species level for all species of the *Azospirillum* cluster (Stoffels et al., 2001). Using fluorescently labeled diagnostic oligonucleotide probes, direct species identification of single cells, as well as *in situ* localization studies, is possible.

DIFFERENTIATION OF THE GENUS *AZOSPIRILLUM* FROM OTHER GENERA

Table BXII.α.4 indicates the characteristics of *Azospirillum* that distinguish it from other diazotrophic bacteria belonging to the *Alphaproteobacteria*.

8. Trace element solution SL-6 (Pfennig, 1974) contains (g/l of distilled water): ZnSO₄·7H₂O, 0.1; MnCl₂·4H₂O, 0.03; H₃BO₃, 0.3; CoCl₂·5H₂O, 0.2; CuCl₂·2H₂O, 0.01; NiCl₂·6H₂O, 0.02; and Na₂MoO₄·2H₂O, 0.03.

TAXONOMIC COMMENTS

In 1921–1922, Beijerinck observed extensive development of a spirillum-like bacterium in nitrogen-deficient D-glucose and mannitol solutions that had been inoculated heavily with garden soil or soil from a sand bed. Although the organism grew well at first, it was later displaced by competitive growth of *Azotobacter* and *Clostridium*. However, when calcium malate or lactate was employed as the carbon source instead of the carbohydrates, the organism grew well and was not overgrown by other nitrogen fixers. Beijerinck found that partially purified cultures of the spirillum exhibited increases in nitrogen at the expense of malate, whereas cultures lacking the spirillum failed to show such increases. Pure cultures of the spirillum failed to grow in the absence of a source of fixed nitrogen, and Beijerinck suggested that good growth in partially purified cultures might be attributable to the microaerophilic nature of the spirillum, as suggested by microaerotactic band formation in wet mounts. In general, cells cultured in sugar media were plump, curved rods containing many lipoidal droplets, which sometimes distorted the shape of the cells. On malate or lactate agar, the cells tended to be thinner and straighter, while in dilute bouillon they exhibited a distinct spirillum shape with one or more helical turns. Because of the ease of cultivation on salts of organic acids, as well as the spirillum shape exhibited under certain conditions, Beijerinck considered the organism to be a member of the genus *Spirillum* and to be a bridging organism linking the genus *Spirillum* with the genus *Azotobacter*. He initially named the organism *A. largimobile*, but later renamed it *Spirillum lipoferum* (Beijerinck, 1925). Later studies of *Spirillum lipoferum* by Schröder (1932) also failed to demonstrate N₂ fixation by pure cultures, and the organism was forgotten for many years except for a few scattered reports. However, in 1963 Becking isolated an organism resembling *Spirillum lipoferum* that showed uncontested nitrogenase activity. Finally, the discovery of the association of such organisms with plant roots (Döbereiner and Day, 1976) caused much interest in *Spirillum lipoferum* and led to detailed taxonomic studies. Tarrand et al. (1978) reserved Beijerinck's specific epithet *lipoferum* for their DNA homology group II (i.e., *A. lipoferum*), because this group seemed to correspond in more ways to Beijerinck's description of *Spirillum lipoferum*, particularly with regard to growth with glucose or mannitol and to formation of spirillum-shaped cells under certain conditions. Since Beijerinck's strains no longer exist, however, their correspondence with homology group II cannot be estimated with certainty.

Analysis of rRNA cistrons indicates that the genus *Azospirillum*

TABLE BXII.α.3. Oligonucleotide sequence probes used for phylogenetic studies of *Azospirillum* species^a

Name of the probe	Sequence of nucleotides	Species-specific target	TD (°C) ^b
AA 23S rRNA	5'-ACA CCT CCA TGG CAC AC-3'	<i>A. amazonense</i>	54
AA/AB 16S rRNA	5'-CGT CCG ATT AGG TAG T-3'	<i>A. amazonense</i> and <i>A. brasilense</i>	48
AB 23S rRNA	5'-GGG TCC CCA GCC GGG C-3'	<i>A. brasilense</i>	60
AH 23S rRNA	5'-TCG CCG CAG CAC GCT-3'	<i>A. halopraeferens</i>	52
AI 23S rRNA	5'-GCA TAC TGG TTT TCA G-3'	<i>A. irakense</i>	46
AI 16S rRNA	5'-CGT CTG ATT AGG TAG T-3'	<i>A. irakense</i>	46
AL 23S rRNA	5'-TAT AAG GCG GGG CTA-3'	<i>A. lipoferum</i>	46
AL 16S rRNA	5'-CGT CGG ATT AGG TAG T-3'	<i>A. lipoferum</i>	48
Adoeb94	5'-CGT GCG CCA CTG TGC CGA-3'	<i>A. doebereineriae</i>	46 ^c
Adoeb587	5'-ACT TCC GAC TAA ACA GGC-3'	<i>A. doebereineriae</i>	46 ^c

^aAdapted from Kirchhof et al., 1997b.

^bTemperature of hybridization should be 5°C below the T_D.

^cIt is recommended that formamide (30% v/v) be added to the standard hybridization buffer.

TABLE BXII.α.4. Differential characteristics of the genus *Azospirillum* and other diazotrophic bacteria belonging to the *Alphaproteobacteria*^a

Characteristic	<i>Azospirillum</i>	<i>Aquaspirillum peregriinum</i>	<i>Rhodospirillum rubrum</i>	<i>Magnetospirillum magnetotacticum</i>	<i>Azorhizobium caulinodans</i>	<i>Xanthobacter autotrophicus</i>	<i>Beijerinckia</i>
<i>Cell shape:</i>							
Vibrioid	+	—	—	—	—	—	— ^b
Helical	— ^c	+	+	+	—	—	—
Cell diameter (μm)	0.6–1.7	0.5–0.7	0.8–1.0	0.2–0.4	0.5–0.6	0.4–0.8	0.5–1.5
Motility	+	+	+	+	+	— ^d	D
Predominant type of flagellation	MP, L	BT	BT	BS	PT, L	±	PT
Nitrogenase activity	+	+	+	+	+	+	+
Nitrogen fixed only under microaerophilic conditions	+	+	+	+	+	+	—
Known to be plant-associated nitrogen fixers	+	—	—	—	+	+	—
Photoautotrophic	—	—	+	—	—	—	—
Root or stem hypertrophies produced	—	—	—	—	+	—	—
Mol% G + C of DNA	64–71	60–64	64–66	65	66–68	65–70	55–61

^aSymbols: +, typically positive; —, typically negative; ±, differ among strains; D, differs among species; MP, monopolar single flagellum; BT, bipolar tuft; BS, bipolar single flagellum; PT, peritrichous; L, lateral flagella.

^b*Beijerinckia* may be straight, slightly curved, or pear shaped.

^cA few helical cells can be observed in *A. halopraefens*.

^dMotility depends on the growth conditions (substrate and age).

belongs to the α -subclass of *Proteobacteria* (De Ley, 1992). This subclass, proposed by Woese et al. (1984b), corresponds to the rRNA superfamily IV described by De Ley (1978) and De Smedt et al. (1980). The majority of the nitrogen-fixing bacteria occur as six major groups within the α subclass, with three of them containing most of the known diazotrophic bacteria. The first cluster exhibits 75°C $T_{m(e)}$ and includes the endophytic diazotroph *Gluconacetobacter diazotrophicus* (Yamada et al., 1998a) (formerly *Acetobacter diazotrophicus*) and the first nitrogen-fixing bacterium from the genus *Gluconacetobacter* described (Gillis et al., 1989). The second cluster is formed by *Zymomonas*, *Rhizomonas*, and *Sphingomonas*. A nitrogen-fixing bacterium isolated from rice plants has been identified as *Pseudomonas paucimobilis* (Bally et al., 1983) and is linked to the *Sphingomonas paucimobilis* rRNA branch at $T_{m(e)}$ of 76°C (Gillis and Reinhold-Hurek, 1994). The third cluster comprises the nitrogen-fixing bacteria of the genus *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, which are linked at $T_{m(e)}$ of 74.4°C to other non-nitrogen-fixing bacteria of the class. A small cluster is formed by the genera *Rhodobacter* and *Paracoccus* and is linked to the rRNA branch at $T_{m(e)}$ of 67°C. In this cluster the diazotrophic species *Rhodobacter capsulatus* is found. The last two clusters are formed by diazotrophic bacteria that are linked to the main rRNA branch of this class at very similar $T_{m(e)}$ values. The first cluster is formed by *Bradyrhizobium*, *Azorhizobium*, *Beijerinckia*, and *Xanthobacter* in addition to *Rhodopseudomonas*, and is linked to the rRNA branch at 70.4°C $T_{m(e)}$, while the last one—formed by *Azospirillum*, *Rhodospirillum*, *Aquaspirillum peregriinum*, and *Magnetospirillum magnetotacticum*—links at 71°C $T_{m(e)}$. Because the last cluster contains the taxa most closely related to *Azospirillum*, their phenotypic and genetic characteristics should be compared with those of the genus *Azospirillum*.

Aquaspirillum peregriinum exhibits phenotypic characters in common with *Azospirillum*: shows nitrogenase activity under microaerophilic conditions; has cell diameter quite similar to that of some *Azospirillum* species but differs from the latter by having helical cells with flagella in bipolar tufts; a mol% G + C of 60–64; an aquatic habitat rather than an association with plants. A relationship between *Azospirillum* and *Rhodospirillum* is indicated by the 16S rRNA cistron analysis where both genera are linked at 71°C $T_{m(e)}$ within the *Alphaproteobacteria*. Certain phenotypic

similarities are also shared specially by the species *A. lipoferum* and *R. rubrum*. The most striking similarity concerns the activation factor for the Fe protein of nitrogenase. This factor has so far been found only in *Azospirillum* and *Rhodospirillum* (Ludden et al., 1978). It is also required for activation of the Fe protein of *A. brasilense* but not for that of *A. amazonense* (Döbereiner and Pedrosa, 1987). *A. lipoferum* and *R. rubrum* share a growth requirement for biotin. Both *Azospirillum* and *R. rubrum* form a pink or red pigment when grown in the dark under aerobic conditions and form intracellular poly- β -hydroxybutyrate. Despite these similarities, there are some significant differences between the two species. *Azospirillum* is not phototrophic, moreover, *R. rubrum* possesses bipolar tufts of flagella and has cells that are thinner and more helical than those of *Azospirillum*. The mol% G + C of the DNA of *R. rubrum* is 64–66, in contrast to the value of 69–70 for *Azospirillum lipoferum*. The complete 16S rRNA gene sequence of all six *Azospirillum* species has shown that *A. amazonense* and *A. irakense* form a subcluster with *Rhodocista centenaria* (basonym *Rhodospirillum centenum*) (Ben Dekhil et al., 1997a). This *Rhodospirillum* species is also a nitrogen-fixing, nonsulfur, purple phototrophic bacterium, accumulates poly- β -hydroxybutyrate, and has a mol% G + C of the DNA of 68.3, very close to that of *A. amazonense* (67–68); however, it has the ability to form cytoplasmic “R” bodies (Favinger et al., 1989), a characteristic not present in *Azospirillum*.

The species *Magnetospirillum magnetotacticum* (formerly *Aquaspirillum magnetotacticum*), and members of the phototrophic genus *Rhodospirillum* are the nearest relatives of the genus *Azospirillum*, based on 16S rRNA gene sequence analysis (Xia et al., 1994; Ben Dekhil et al., 1997a). Cells from *M. magnetotacticum* are obligately microaerophilic, accumulate poly- β -hydroxybutyrate, fix nitrogen only under microaerophilic conditions, and have a mol% G + C of the DNA of 65 that is in the same range as that of *Azospirillum* (64–71). However, the smaller cell size, type of flagellation, and formation of coccoid bodies 3–4 weeks after growth, as well as the occurrence of magnetite-containing “magnetosomes”, excludes it from the genus *Azospirillum*.

The nitrogen fixer *Azorhizobium caulinodans* resembles *Azospirillum* in being a free-living, obligately aerobic, small rod that fixes N₂ microaerobically, as well as being a plant-associated dia-

zotroph. However, the host–bacterium interaction (stem nodule formation) is far more highly specialized than that of *Azospirillum* for symbiotic nitrogen fixation. In addition, *Azorhizobium caulinodans* forms a main cluster with *Xanthobacter autotrophicus*, *Beijerinckia*, and *Bradyrhizobium* which links at 70.4°C $T_{m(e)}$ to the root of the *Alphaproteobacteria*; *Azospirillum* links at 71°C $T_{m(e)}$.

Xanthobacter autotrophicus resembles some species of *Azospirillum* in being a microaerophilic nitrogen fixer, having an association with rice plants, and having a mol% G + C of the DNA (65–70) that is similar to that of *Azospirillum*. However, the cells differ by being smaller (and under certain conditions exhibiting branching), nonmotile, and forming a yellow pigment.

The genus *Beijerinckia* lies within the same *Alphaproteobacteria* group and forms a subcluster together with *Bradyrhizobium*, *Azorhizobium*, and *Xanthobacter* which links to the main RNA branch at 71°C $T_{m(e)}$, according to the rRNA cistron dendrogram for this class (De Ley, 1992). Although *Beijerinckia* cells are straight or slightly curved rods with a diameter similar to that of *Azospirillum*, other characteristics such as the ability to fix nitrogen aerobically, occurrence as a free-living bacterium in soil, and low mol% G + C values (55–61) prevent the inclusion of this genus in *Azospirillum*. Nevertheless, the *Azospirillum largimobile* conglomerate cells show a striking resemblance to cells of *Beijerinckia* species.

By DNA–DNA hybridization, *A. brasilense* strains exhibit a continuum of similarity values with the type strain, ranging from ~70 to 100%. Within the species *A. lipoferum*, strains exhibit similarity values of 70–76% with the type strain. In the case of *A. amazonense*, similarity values range from 56 to 100% with the type strain. A DNA–DNA homology value of 100% was observed for hybridization of one strain of *A. halopraeferens* with the type strain. Within the species *A. irakense*, strains exhibited similarity values of 71–97% with the type strain. For *A. largimobile*, the similarity value was 65% (one isolate) with the type strain.

From DNA–DNA hybridization studies using a membrane-filter competition method, *A. lipoferum* and *A. brasilense* initially were reported to exhibit similarity values of 31–34% (Tarrand et al., 1978). Use of the S1 nuclease method, however, indicated that *A. lipoferum* ATCC 29707 and *A. brasilense* ATCC 29145 exhibited lower similarity values (14–17%) to each other (Falk et al., 1985). A much lower degree of similarity (2–6%) was observed when both species were hybridized against *Azospirillum amazonense* ATCC 35119 (Falk et al., 1985). In the case of *Azospirillum halopraeferens* (LMG 7108), a similarity value of <25% was observed between the three species (Reinhold et al., 1987). A low degree of similarity (4–11%) was also exhibited by the type strain of *Azospirillum irakense* (CIP 103311) to the four above-mentioned species (Khammas et al., 1989). The recently described *Azospirillum largimobile*—formerly *Conglomeromonas largimobilis*—exhibited a very low degree of similarity to *A. amazonense* (5–7%) and *A. brasilense* (13–14%) (Falk et al., 1986); however, it showed a high level of similarity (40–47%) to *Azospirillum lipoferum*. Because of this similarity and other morphological characteristics, Falk et al. (1986) considered strains from this group as belonging to *Azospirillum lipoferum*. Based on the binary se-

quence similarity values of 16S rRNA genes, Ben Dekhil et al. (1997a) demonstrated that this new species shared 97.1% similarity with *A. lipoferum* but much less with *A. brasilense* (95.2%) and *A. amazonense* (91.7%). Although this difference is small, the authors proposed *A. largimobile* as a new species based on the DNA hybridization data.

An analysis based on the similarity values of the 16S rRNA genes of *Azospirillum* species versus the type species *A. lipoferum* indicates the occurrence of two subgroups within the genus. One includes *A. lipoferum*, *A. brasilense*, *A. halopraeferens*, *A. doebereineriae*, and *A. largimobile*; the other includes *A. amazonense* and *A. irakense* (Ben Dekhil et al., 1997a). In addition, this study showed that *A. brasilense* is most closely related to *A. lipoferum*, followed by *A. halopraeferens* and *A. largimobile*. Fani et al. (1995b) also have observed that *A. lipoferum* and *A. brasilense* are very closely related species, whereas *A. amazonense* was the most divergent. Xia et al. (1994) showed that all type strains of *Azospirillum* formed a deep-branching clade within the *Alphaproteobacteria* when the 16S rDNA sequences were compared. The most recent comprehensive 16S rDNA sequence study of Stoffels et al. (2001) also confirmed a high degree of relatedness among *Azospirillum* species and that they form a cohesive phylogenetic cluster within the *Alphaproteobacteria*. A strain-specific molecular identification of *Azospirillum* strains is possible using RFLP analysis of the whole genome using rarely cutting restriction nucleases and pulsed-field gel electrophoresis (Gündisch et al., 1993).

ACKNOWLEDGMENTS

This chapter is dedicated to the memory of the late Dr. Johanna Döbereiner, the Brazilian soil microbiologist, who, during a long and distinguished career, identified and characterized many novel nitrogen-fixing bacteria.

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *AZOSPIRILLUM*

The differential characteristics of the species of *Azospirillum* are indicated in Table BXII.α.5. Other characteristics of the species are presented in Table BXII.α.6.

TABLE BXII.α.5. Characteristics differentiating the species of the genus *Azospirillum*^{a,b}

Characteristics	<i>A. lipoferum</i>	<i>A. amazonense</i>	<i>A. brasilense</i>	<i>A. doebereineriae</i>	<i>A. halopraeferens</i>	<i>A. irakense</i>	<i>A. largimobile</i>
Cell width, µm	1.0–1.7	0.8–1.0	1.0–1.2	1.0–1.5	0.7–1.4	0.6–0.9	0.7–1.5
Enlarged, pleomorphic cells develop in alkaline media	+	–	–	+	+	+	+
Growth with 3% NaCl	d	–	–	–	+	+	–
Biotin requirement	+	–	–	–	+	–	+
Pectin hydrolysis	–	–	–	nt	–	+	–
Optimal growth temperature, °C	37	35	37	30	41	33	28
<i>Carbon sources:</i>							
Glucose	+	+	–	d	–	+	+
Mannitol	+	–	–	+	+	–	+
Glycerol	+	–	+	+	+	–	+
Sucrose	–	+	–	–	–	+	–
<i>Hybridization with probes:</i> ^{c,d}							
AZO 23S rRNA	+	+	+	na	+	+	na
AL 23S/16S rRNA	+	–	–	na	–	–	na
AB 23S rRNA	–	–	+	na	–	–	na
AA 23S rRNA	–	+	+	na	–	–	na
AA/AB 16S rRNA	–	+	+	na	–	–	na
AH 23S rRNA	–	–	–	na	+	–	na
AI 23S/16S rRNA	–	–	–	na	–	+	na
Adoeb94	–	–	–	+	–	–	–
Adoeb587	–	–	–	+	–	–	–

^aSymbols: +, positive; –, negative; d, differs among strains; na, probes not available; nt, not tested.

^bData from Magalhães et al. (1983); Krieg and Döbereiner (1984); Reinhold et al. (1987); Khammas et al. (1989); and Ben Dekhil et al. (1997a).

^cData from Kirchhof et al. (1997b).

^dData from Eckert et al. (2001).

List of species of the genus *Azospirillum*

1. ***Azospirillum lipoferum*** (Beijerinck 1925) Tarrand, Krieg and Döbereiner 1979, 79^{AL} (Effective publication: Tarrand, Krieg and Döbereiner 1978, 978 (*Spirillum lipoferum* Beijerinck 1925, 353.)

li.po'fe.rum. Gr. n. *lipus* fat; L. v. *fero* to carry; M.L. adj. *lipoferus* fat bearing.

The characteristics are as described in Tables BXII.α.4, BXII.α.5, and BXII.α.6. The morphological characteristics are depicted in Figs. BXII.α.4 and BXII.α.5. The characteristic pleomorphism seen in malate medium does not occur in D-glucose medium. Cells of certain strains become much thinner and very long in malate medium. Use of D-glucose as the sole carbon source for nitrogen-fixation-dependent growth is more efficient at pH 5.5–6.5 than at pH 7.0–7.5.

All strains stain uniformly Gram negative when cultured in MPSS broth. Most strains stain uniformly Gram negative when cultured for 48–72 h on MPSS agar but a few strains exhibit a small proportion of cells that are resistant to Gram decolorization.

Colonies are not slimy on BMS agar. When 0.5% D-glucose is added to the medium, some strains form large, slimy, white colonies. Star-shaped colonies have also been observed for some strains grown on BMS agar plates (Fig. BXII.α.11).

Aerobic growth in liquid media containing a source of fixed nitrogen often shows extensive clumping, particularly in defined media. No growth occurs anaerobically in the absence of a source of fixed nitrogen. However, nitrogen fixation can be observed in liquid medium containing glu-

tamate when the cells reach an optical density of approximately 1.0 and all available nitrogen is consumed.

Strains of *A. lipoferum* that are able to grow lithoautotrophically with H₂ contain an uptake hydrogenase as well as RubPcase. They can be categorized as aerobic hydrogen-oxidizing bacteria.

Physiological and nutritional characteristics of the species are presented in Tables BXII.α.5 and BXII.α.6. Variants with decreased fermentative ability may arise but continue to require biotin, use D-glucose as a sole carbon source for N₂-dependent growth, and exhibit the characteristic pleomorphism associated with this species in malate medium.

Strains of this species have been found colonizing several plants including cereals, forage grasses, vegetables, legumes, and the fruits of banana and pineapple plants.

The mol% G + C of the DNA is: 69–70 (*T_m*).

Type strain: BR11080, Sp 59b, ATCC 29707, DSM 1691.

GenBank accession number (16S rRNA): M59061.

Additional Remarks: Reference strains: ATCC 29708 (BR11115; Sp Rg 20a), ATCC 29709 (BR11084; Sp Br 17), ATCC 29731 (BR11087; Sp Rg 6xx). The accession numbers of the 16S rDNA sequences from reference strains ATCC 29708 and ATCC 29731 deposited at the EMBL are X79729 and X79730, respectively (Fani et al., 1995a). Other 16S sequences from WO3 and NCIMB 11861 have received the accession numbers X79741 (Fani et al., 1995a) and Z29619 (Xia et al., 1994), respectively.

2. ***Azospirillum amazonense*** Magalhães, Baldani, Souto, Kuykendall and Döbereiner 1984, 355^{VP} (Effective publication: Magalhães, Baldani, Souto, Kuykendall and Döbereiner 1983, 417.)

TABLE BXII.α.6. Other characteristics of the species of the genus *Azospirillum*^{a,b}

Characteristics	<i>A. lipoferum</i>	<i>A. brasilense</i>	<i>A. amazonense</i>	<i>A. halopraeferens</i>	<i>A. irakense</i>	<i>A. largimobile</i>	<i>A. doebereineriae</i>
Flagella arrangement	MP, L	MP, L	MP	MP	MP, L	MP, L	MP, L
Colony type on:							
BMS agar medium	pink, raised, curled scarlet	pink, raised, curled scarlet	white, flat raised margin	ng	nd	nd	nd
Congo red medium			nd	ng	nd	scarlet	scarlet
Dissimilation of:							
NO ₃ ⁻ to NO ₂ ⁻	+	+	d	+	d	+	+
NO ₂ ⁻ to N ₂ O	d	d	—	+	—	—	+
NO ₃ ⁻ -dependent anaerobic growth	+	+	—	+	—	—	+
pH range for growth	5.7–6.8	6.0–7.8	5.7–6.5	6.8–8.0	5.5–8.5	nd	6.0–7.0
Acid from:							
Glucose	+	—	—	—	—	+	d
Fructose	+	—	—	+	—	+	+
Acidification of:	+	—	—	—	—	nd	nd
peptone-based glucose broth							
Sole carbon sources (auxanographic method):							
Succinate, malate, lactate, fumarate	+	+	+	+	+	nd	+
Gluconate	+	+	—	+	—	—	+
Citrate	+	+	—	v	—	+	—
α-Ketoglutarate	+	—	—	nd	v	nd	—
myo-Inositol	v	+	+	—	—	—	—
D-Sorbitol	+	—	—	—	—	+	+
D-Ribose	+	—	+	+	v	+	—
L-Rhamnose	+	v	+	v	+	—	—
Fructose	+	+	+	+	v	+	+
Maltose	—	—	+	nd	+	—	—
Lactose	—	—	v	nd	+	—	—
D-Mannose	v	—	+	+	+	—	—
Habitat:							
Soil and tissues mainly of nonlegumes	+	+	+				
Roots of <i>Leptochloa fusca</i> (L.) grown in saline-sodic soil, Pakistan				+			
Soil and roots of rice plants grown in Iraq					+		
Freshwater lake, Australia						+	
Soil and roots of <i>Miscanthus</i> , Germany							+
% similarity in 16S rRNA vs. <i>A. lipoferum</i> ^{c,d}	100	98.1	94	97.2	94	97.1	96.6
Mol% G + C of DNA	69–70	70–71	67–68	69–70	64–67	70	69.6–70.7

^aSymbols: see standard definitions; nd, not determined; ng, no growth.^bData from Magalhães et al. (1983); Krieg and Döbereiner (1984); Reinhold et al. (1987); Khammas et al. (1989); and Ben Dekhil et al. (1997a).^cBased on data from Ben Dekhil et al. (1997a).^dBased on data from Eckert et al. (2001).

am.a.zo.nen'se. M.L. adj. *amazonense* pertaining to the Amazon region of Brazil, South America.

The characteristics are as described in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. Colonies on potato agar medium (BMS) containing malate plus sucrose as sole carbon source are 0.5 cm in diameter and are flat with elevated borders. The colonies become bigger and flat with well-delineated borders when both carbon sources are replaced by D-glucose (Fig. BXII.α.12) and become smaller, wet, and smooth when sucrose is omitted from the BMS medium (Fig. BXII.α.12), indicating the sensitivity of this species to alkaline conditions. This is also the reason why only a scant pellicle formation is observed in semi-solid NFB medium containing malate but not with sucrose as carbon source. The structure

of the colonies on BMS plates tends to disappear after a long period of incubation. Very dry, white colonies with well-delineated borders are also observed on BMS plates containing sucrose as sole carbon source. Cells remain vibrioid in semi-solid LGI medium with spinning movement. However, long periods of incubation in semi-solid LGI medium allow formation of conglomerate cells similar to cysts observed for *A. lipoferum*. This may be due to the dryness of the medium.

Significant amounts of PHB granules are observed under N₂ growth conditions but much less in the presence of NH₄⁺. Nitrogenase activity is more sensitive than in *A. lipoferum* and *A. brasilense*, and *A. amazonense* has a doubling time of 10 hours, in contrast to 5–6 h for *A. lipoferum* and *A. brasilense* at low pO₂.

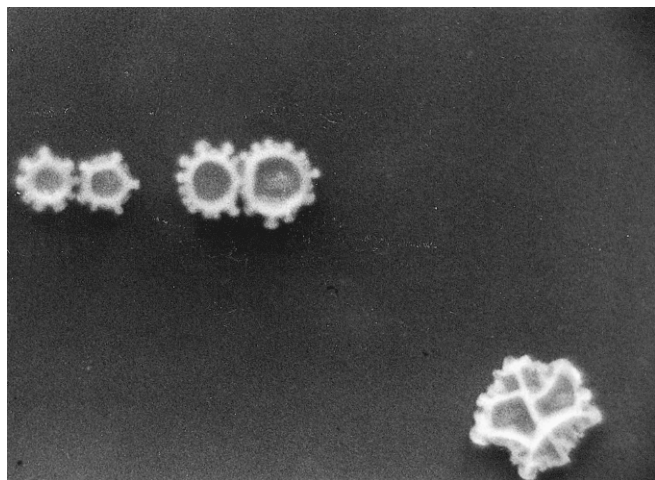


FIGURE BXII.α.11. Colonies of *Azospirillum lipoferum* grown on BMS agar plate for 48 h at 30°C showing starlike forms.

Good aerobic growth occurs in liquid LGI medium containing KNO_3 as nitrogen source but not with NH_4Cl . There is no clumping and the pH remains around 6.0. No growth occurs aerobically in the absence of a source of fixed nitrogen, as observed for the other species. However, nitrogen fixation can be observed in liquid medium containing glutamate when the cells reach optical density around 1.0 and all available nitrogen is consumed.

Fatty acid analysis showed higher amounts of $\text{C}_{16:0}$ as compared to the $\text{C}_{18:1}$ nonhydroxy fatty acids. This species has been isolated from roots of maize, sorghum, rice and wheat plants, as well as forage grasses grown around Brazil. It was also isolated from roots and stems of sugarcane plants grown in Brazil and from sugarcane roots grown in Hawaii

and Thailand. It was also found associated with roots of palm trees from the Amazon region and more recently from pineapple and banana fruits. The root interior of rice, sorghum, and maize was predominantly colonized by *A. amazonense* strains that do not accumulate nitrite rather than those that produce considerable amounts of nitrite, which afterwards is reassimilated.

The mol% G + C of the DNA is: 67–68 (T_m).

Type strain: BR 11142, Am14, Y1, ATCC 35 119, DSM 2787.

GenBank accession number (16S rRNA): Z29616, X79735.

Additional Remarks: Reference strains: ATCC 35120 (BR 11140; Am18; Y2); ATCC 35121 (BR 11141; Am30; Y6). The accession number for the reference strain ATCC 35120 is X79742 (Fani et al., 1995a).

3. ***Azospirillum brasilense*** Tarrand, Krieg and Döbereiner 1979, 79^{AL} (Effective publication: Tarrand, Krieg and Döbereiner 1978, 979.)

bra.si.len'.se. M.L. adj. *brasilense* pertaining to the country of Brazil, South America.

The characteristics are as described for the genus and as given in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. In semisolid, nitrogen-free, malate medium cells remain mainly vibrioid even when the cultures become alkaline, in contrast to the cells of *A. lipoferum*, *A. irakense*, *A. halopraeferens*, and *A. largimobile*. Some encapsulated (C) forms (Fig. BXII.α.6) may occur, especially in older cultures. C forms occur in abundance in colonies grown on the surface of nitrogen-free media, in association with plant callus cultures, in grass seedlings inoculated with this species, or under stress conditions. Flocculated cells of *A. brasilense* strain Cd grown under conditions of stress accumulate up to 60–65% of total dry weight as PHB.



FIGURE BXII.α.12. Colonies of *Azospirillum amazonense* strain ATCC 35119 grown on BMS agar plates containing only malate (upper left), malate plus sucrose (lower left), or only glucose (right) as carbon source for 48 h at 30°C. (Reproduced with permission from J. Döbereiner et al., Brasília: Embrapa-SPI: Itaguaí, RJ: Embrapa-CNPAB, 1995.)

All strains stain uniformly Gram negative when cultured in MPSS broth. Most strains exhibit a small proportion of cells showing resistance to Gram decolorization when cultured on MPSS agar for 48–72 h; this Gram variability may be related to the occurrence of encapsulated forms.

Certain strains and variants of *A. brasilense* form colonies that have a much deeper shade of pink than is usually the case. Carotenoid content has been identified as responsible for the observed characteristic in strain Cd.

Aerobic growth in liquid media containing a source of fixed nitrogen is usually homogeneous and turbid, without clumping. No growth occurs aerobically in the absence of a source of fixed nitrogen. Cells are able to fix nitrogen in liquid aerated medium when pre-grown in the presence of glutamate, up to an optical density around 1.0, and no inorganic nitrogen is available. Strains of this species have been found colonizing several plants including cereals, forage grasses, vegetables, legumes, and banana plants.

The mol% G + C of the DNA is: 70–71 (T_m).

Type strain: BR11001, Sp 7, ATCC 29145, DSM 1690.

GenBank accession number (16S rRNA): X79739.

Additional Remarks: Reference strains: ATCC 29710 (BR11002; Cd); ATCC 29711 (Sp 35). The accession number is Z29617 (Xia et al., 1994) for strain NCIMB 11860.

4. **Azospirillum doebereineriae** Eckert, Weber, Kirchhof, Halbritter, Stoffels and Hartmann 2001, 24^{VP}
doeb' er.ein.er.ae. N.L. gen. fem. n. *doebereineriae* of Döbereiner, in honor of Johanna Döbereiner, who isolated and characterized many *Azospirillum* spp., and other diazotrophic plant-associated bacteria.

The characteristics are as described for the genus and as given in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. Cells are Gram negative, oxidase, catalase, and urease positive, curved rods or S shaped. Long cells are observed, especially in alkaline, semi-solid, Nfb medium and motile with a winding or snake-like movement. Optimal growth and nitrogen fixation occur in semi-solid Nfb medium at 30°C and pH adjusted to 6.5. Production of poly-β-hydroxybutyrate is also observed. No growth or nitrogen fixation occurs in liquid Nfb medium. So far, it has been isolated only from the root of *Miscanthus sinensis* cv. "Giganteus" and *Miscanthus sacchariflorus* and also in the rhizosphere soil of these plants grown in Freising, Germany.

The mol% G + C of the DNA is: 70.7 (T_m).

Type strain: GSF71, DSM 13131.

GenBank accession number (16S rRNA): AJ238567.

5. **Azospirillum halopraeferens** Reinhold, Hurek, Fendrik, Pot, Gillis, Kersters, Thielmans and De Ley 1987, 48^{VP}
ha.lo.prae' fe.rens. Gr. n. *hals* salt, the sea L. v. *praeferre* to prefer; M.L. part. adj. *halopraeferens* salt preferring.

The characteristics are as described for the genus and as given in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. Cells are Gram negative, oxidase, and urease positive, vibrioid to S shaped. A few long, helical cells are observed in older, more alkaline medium. Cells are motile in liquid media with rapid corkscrew-like motion. Optimal growth and nitrogen fixation occur in semi-solid SM medium containing 0.25% NaCl at 41°C and pH adjusted to 7.2. Strains are able to grow with 3% NaCl but no nitrogenase activity is observed. No colonies

are formed in BMS or Congo Red medium. Good growth is observed in tryptic soy agar where the colonies are cream-colored, circular and flat with an entire margin.

Production of poly-β-hydroxybutyrate is also observed. No growth or nitrogen fixation occurs in liquid SM medium.

Strains of this species show higher osmotolerance than *A. lipoferum*, *A. brasilense*, *A. amazonense*, *A. doebereineriae*, and *A. largimobile*. Growth and nitrogen fixation is stimulated by glycine betaine, as found in *A. brasilense* but not in *A. amazonense* or *A. lipoferum*.

This species seems to have a very restricted habitat. So far, it has been isolated only from the root surface of Kallar grass (*Leptochloa fusca*) grown in saline-sodic soils in Punjab, Pakistan. Attempts to isolate this species from various plants grown in saline-affected soils around Brazil have been unsuccessful.

The mol% G + C of the DNA is: 69–70 (T_m).

Type strain: Au 4, DSM 3675, LMG 7108.

GenBank accession number (16S rRNA): Z29618, X79731.

6. **Azospirillum irakense** Khammas, Ageron, Grimont and Kaiser 1991, 580^{VP} (Effective publication: Khammas, Ageron, Grimont and Kaiser 1989, 688.)
i.ra.ken' se. L. neut. adj. *irakense* pertaining to the country of Iraq.

The characteristics are as described for the genus and as given in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. The morphological characteristics of the cells in nutrient broth are shown in Fig. BXII.α.7. The cells are Gram-negative, oxidase and catalase positive, curved, vibrioid to S-shaped rods and can reach a length of 30 μm when grown in nutrient broth. Cells are motile with winding or snake-like movements by a single polar flagellum. Colonies are translucent, glistening, convex with regular margin, 1 mm in diameter after 4 d on Nfb medium containing yeast extract (20 mg/l) or on AAM agar medium. On tryptic soy agar, colonies are translucent with an opaque center, convex with regular margin, and 3 mm in diameter after 4 d.

Nitrogen fixation occurs only under microaerobic conditions and cells grow well under air atmosphere in the presence of a nitrogen source such as an ammonium salt. Intracellular granules (PHB) are observed.

The physiological and nutritional characteristics are presented in Tables BXII.α.5 and BXII.α.6. Growth occurs in the presence of up to 3% NaCl, but acetylene reduction is optimal when the NaCl concentration is less than 0.01%. All strains hydrolyze esculin and pectin but not hydroxyquinoline-β-glucuronide. Pectin can support growth and nitrogen fixation could be detected in modified, semisolid, AAM medium after 5–11 days of incubation provided that a small amount of fixed nitrogen as starter is present (Khammas and Kaiser, 1991). The species so far has only been isolated from rhizosphere soil and roots of rice plants grown in the region of Diwaniyah in Iraq.

The mol% G + C of the DNA is: 64–67 (T_m).

Type strain: KBC1, CIP 103311, DSM 11586.

GenBank accession number (16S rRNA): Z29583, X 79737.

7. **Azospirillum largimobile** (Skerman, Sly and Williamson 1983) Ben Dekhil, Cahill, Stackebrandt and Sly 1997b, 915^{VP} (Effective publication: Ben Dekhil, Cahill, Stacke-

brandt and Sly 1997a, 74) (*Conglomeromonas largomobilis* subsp. *largomobilis* Skerman, Sly and Williamson 1983, 300.) *lar.gi.mo'bi.le.* L. adj. *largus* a very slow manner (musical); L. v. *mobile* to move; M.L. adj. *largimobile* moving in a very slow manner.

The characteristics are as described for the genus and as given in Tables BXII.α.4, BXII.α.5, and BXII.α.6, with the following additional features. Strains of this species are Gram negative, oxidase, urease, and aminopeptidase positive, and are weakly positive for catalase and phosphatase. Strains exhibit two phases of growth—unicellular and multicellular. Cells are rod shaped with round or tapered ends and a straight or slightly curved axis in the unicellular phase. Motile cells have mixed flagellation. Multicellular conglomerates are formed, with the cells losing motility under unfavorable conditions. The conglomerate dissociates into single motile cells under suitable conditions, forming clear colonies on agar medium. Colonies on lake water agar become buff colored, opaque, low convex, and round

with an entire edge and smooth surface after 72 h at 28°C. No encapsulated cells are observed.

N₂ fixation and acetylene reduction were observed only under microaerophilic conditions in semi-solid, nitrogen-free, malate medium with biotin. No nitrogen fixation was detected under aerobic conditions in the presence or absence of a nitrogen source such as ammonium salt.

Other physiological and nutritional characteristics are shown in Tables BXII.α.5 and BXII.α.6. Most of these characteristics of *A. largimobile* are similar to those of *A. lipoferum*, except for acid production from salicin and D-xylose, which is only present in the latter. In addition, *A. largimobile* is DNase positive and shows clearing of egg yolk agar. Strains are able to grow (slowly) in the presence of 2% NaCl. This species is composed of only two strains isolated from fresh lake water in Australia.

The mol% G + C of the DNA is: 69.6–70.8 (T_m).

Type strain: ACM 2041, DSM 9441, UQM 2041.

GenBank accession number (16S rRNA): X90759.

Other Organisms

A noncellulolytic bacterium (strain Mc-2s) having the characteristics of the genus *Azospirillum* was isolated from cellulolytic, nitrogen-fixing, mixed cultures by Wong et al. (1980a). The strain exhibited a biotin requirement, was pleomorphic in nitrogen-deficient malate medium, and could utilize mannitol and α-ketoglutarate. Although these are characteristics of *A. lipoferum*, the strain failed to use D-glucose as a sole carbon source for N₂-dependent growth and did not acidify D-glucose or ribose media. The strain may be a variant of *A. lipoferum*. In another study, Franche and Elmerich (1981) found *Azospirillum* strains K67 and KR77 to be capable of glucose utilization, yet they had no biotin requirement; they were provisionally classified as *A. brasilense*. A third strain, SpN, appeared to be an *A. lipoferum* strain but seemed to require other growth factors in addition to biotin.

Nur et al. (1980) isolated microaerophilic, nitrogen-fixing, vibrioid bacteria having certain characteristics in common with *Azospirillum*. The organisms were associated with grass roots, were vibrioid cells with a single polar flagellum, and could use malate, lactate, arabinose, and galactose, but not mannitol, as sole carbon sources; one isolate could use glucose. Biotin was not required. These strains differed from *Azospirillum* by having a smaller cell diameter (0.5–0.6 μm), by having no lateral flagella, and by forming yellow-pigmented colonies. Their taxonomic placement is uncertain.

Two strains of *Azospirillum* (Am-53 and Al-3) were isolated from soil and plant litter in Göttingen, Germany, as nitrogen-fixing, hydrogen-oxidizing bacteria. These were Gram negative but showed Gram-positive granules and contained lipid droplets. Both had polar flagella and exhibited an active spirillum-like motility. Strain Am-53 (DSM 1727) required biotin, was pleomorphic in nitrogen-deficient malate medium, and in several other characters resembled *A. lipoferum*. Like *A. lipoferum*, it could grow chemolithotrophically on H₂ (Málek and Schlegel, 1981). However, unlike *A. lipoferum*, it could not utilize glucose, malonate, mannitol, or α-ketoglutarate as a sole source of carbon.

Strain Al-3 (DSM 1726) was shorter than *A. lipoferum*. The cells were pleomorphic, but in the presence of 2–3% (w/v) NaCl the cells developed an active spinning motility (around the axis). Morphologically and in several other respects, the strain resembled *A. brasilense*; however, it could grow chemolithotrophically on H₂, could utilize glucose and α-ketoglutarate as a sole carbon source, and could produce acid from glucose aerobically, like *A. lipoferum*. A recent 16S rRNA gene sequence analysis showed that both of these *Azospirillum*-like strains were misclassified. The strain DSM 1726 is closely related to *Agrobacterium tumefaciens*, while DSM 1727 is related to bacteria within the *Gammaproteobacteria* (Xia et al., 1994).

A variant of *Azospirillum halopraeferens* was isolated from roots of rice plants grown in soil with pH 8.0 in India (Balasubramanian and Prabhu, 1989). This isolate grows and fixes nitrogen in semi-solid NFb medium, is highly motile and vibrioid. However, it could grow and fix nitrogen in the presence of 5% NaCl, utilize glucose and sucrose, and acidify peptone-based glucose broth. The ability to use D-glucose and sucrose as carbon sources are characteristics of *Azospirillum irakense*, a diazotroph isolated from rice plants grown in Iraq. Therefore, it seems that the Indian isolate may be more closely related to *A. irakense* than to *A. halopraeferens*.

Two groups of strains showing many morphological and physiological characteristics similar to *A. lipoferum* species were isolated from soil and rice plants grown in North Vietnam (Ngoc Dung et al., 1995). One group (Arm 2-2, GL 1-1) could use D-glucose, sucrose, and α-ketoglutarate, but not mannitol. The other group (strain DA 10-1) also utilized glucose and sucrose, but not mannitol or α-ketoglutarate. Neither group required biotin but formed acid from glucose and scarlet colonies on BMS medium. Additionally, neither group showed positive signals when hybridized with *Azospirillum* probes, and they may represent a new species.

Genus III. *Levispirillum* gen. nov.

BRUNO POT AND MONIQUE GILLIS

Le.vi.spi.ril' lum. L. adj. *laevus* left; M.L. dim. neut. n. *spirillum* small spiral; *Levispirillum* small spiral-shaped bacteria with left-turning helix.

Gram-negative, **rigid, helical cells 0.4–0.7 µm in diameter and 2–22 µm in length**. Helix is left-handed, determined by focusing on the bottom of the cells: the pattern \\\ indicates a counterclockwise (left-handed) helix. **A polar membrane is present under the cytoplasmic membrane**. Intracellular polyhydroxybutyric acid and coccoid bodies are usually formed. **Bipolar tufts of flagella**. Chemoorganotrophic with an oxidative type of metabolism, using oxygen as a terminal electron acceptor. One species grows anaerobically with nitrate as electron acceptor. Acid is formed from fructose aerobically and anaerobically. Oxidase, catalase, and phosphatase positive; sulfatase negative. Indole negative and esculin positive. Gelatin is not liquefied in 4 d at 30°C or in 7 d at 20°C. One species can liquefy gelatin at 20°C after 28 d. Growth factors not required. Nonphototrophic. No growth on casein, starch, and hippurate. Nitrogenase activity can occur

in anaerobic conditions in the presence of ammonium sulfate. No growth at 3% NaCl. Growth on 1% bile and on EMB agar. L-Glutamate, L-asparagine, L-glutamine, and L-proline used as sole C-source; caproate, isocitrate, D-glucose, D-xylose, L-arabinose, and L-tyrosine, L-methionine, L-serine, L-tryptophan, L-isoleucine, and glycine cannot be used as sole C-source. Isolated from pond water and from putrid infusions of freshwater mussels.

The mol% G + C of the DNA is: 60–64.

Type species: Levispirillum itersonii comb. nov. (*Aquaspirillum itersonii* (Giesberger 1936) Hylemon, Wells, Krieg and Jannasch 1973b, 370.)

ACKNOWLEDGMENTS

We sincerely acknowledge Dr. N.R. Krieg for kindly providing the template text upon which this chapter was based.

List of species of the genus *Levispirillum*

1. *Levispirillum itersonii* comb. nov. (*Aquaspirillum itersonii* (Giesberger 1936) Hylemon, Wells, Krieg and Jannasch 1973b, 370.)
i.ter.so'ni.i. M.L. gen. n. *itersonii* of Itersen; named for G. Van Itersen, a Dutch bacteriologist.

The morphological characters are shown in Fig. BXII.β.67 and described in Table BXII.β.83. Optimal growth temperature 32–35°C. Chemotaxonomic and physiological characters are as described in Tables BXII.β.80, BXII.β.81, BXII.β.82, and BXII.β.83. Sole carbon sources are listed in Table BXII.β.79. Ammonium salts can serve as sole nitrogen sources. Nitrate supports either no growth or very scanty growth. In 1957, Williams and Rittenberg established a subspecies, subsp. *vulgatum*, to include those strains that use nitrate; however, this could not be confirmed by Terasaki (1979) for the type strain of the subspecies (ATCC 11331), using the methods of Williams and Rittenberg.

Isolated from pond water and from putrid infusions of freshwater mussels.

The two subspecies, subsp. *itersonii* and subsp. *nipponicum*, as represented by the type strains IFO 15648 and IFO 13615, respectively, have been shown to be genotypically related at the species level (Kawasaki et al., 1997). The type strain of *L. itersonii* subsp. *nipponicum*, as represented by the subculture ATCC 33333^T, however, could not be located in the *Alphaproteobacteria*, but was found to belong to the *Betaproteobacteria*, where it is a member of the family *Comamonadaceae* (Willems, unpublished results).

The mol% G + C of the DNA is: 60–66 (T_m).

Type strain: Giesberger, ATCC 12639, NCIMB 9070, NRRL B-2053.

GenBank accession number (16S rRNA): Z29620.

- a. *Levispirillum itersonii* subsp. *itersonii* subsp. nov. (*Aquaspirillum itersonii* Hylemon, Wells, Krieg and Jannasch 1973b, 370.)

Morphology and characteristics as for the species. Differs from the subsp. *nipponicum* by having a cell diameter of 0.4–0.6 µm and rapid formation of coccoid bodies (within 7 d).

The mol% G + C of the DNA is: 60–64 (T_m).

Type strain: Giesberger, ATCC 12639, NCIMB 9070, NRRL B-2053.

GenBank accession number (16S rRNA): Z29620.

- b. *Levispirillum itersonii* subsp. *nipponicum* subsp. nov. (*Spirillum itersonii* subsp. *nipponicum* Terasaki 1973, 58.)
nip.po'ni.cum. M.L. neut. adj. *nipponicum* pertaining to the country of Japan.

Morphology and characteristics as for the species. Differs from the subsp. *itersonii* by having a cell diameter of 0.5–0.8 µm and a delayed formation of coccoid bodies (~2 weeks before they become predominant).

The mol% G + C of the DNA is: 66 (T_m).

Type strain: KF 8, ATCC 33333, DSM 11590, IFO 13615.

2. *Levispirillum peregrinum* comb. nov. (*Aquaspirillum peregrinum* (Pretorius 1963) Hylemon, Wells, Krieg and Jannasch 1973b, 370.)

pe.re.gri' num. L. neut. adj. *peregrinum* strange, foreign.

The morphological characters are shown in Fig. BXII.β.67 and described in Table BXII.β.83. Optimal growth temperature 32°C. Chemotaxonomic and physiological characters are described in Tables BXII.β.80, BXII.β.81, BXII.β.82, and BXII.β.83. Sole carbon sources are listed in Table BXII.β.79. Ammonium salts can be used as sole nitrogen sources. There are conflicting reports concerning the ability to use nitrate as a sole nitrogen source (Terasaki, 1972, 1979; Hylemon et al., 1973b).

Isolated from a primary oxidation pond and from the putrid infusion of a freshwater mussel.

The two subspecies, subsp. *peregrinum* and subsp. *integrum*, have been shown to be genotypically related at the species level (Pot, 1996; Kawasaki et al., 1997).

The mol% G + C of the DNA is: 60–64 (T_m).

Type strain: ATCC 15387, DSM 1839, NCIB 9435.

- a. *Levispirillum peregrinum* subsp. *peregrinum* subsp. nov. (*Aquaspirillum peregrinum* (Pretorius 1963) Hylemon, Wells, Krieg and Jannasch 1973b, 370.)

Morphology and characteristics as for the species. Differs from the subsp. *integrum* by forming coccoid bodies.

The mol% G + C of the DNA is: 60–62 (T_m).

Type strain: ATCC 15387, DSM 1839, NCIB 9435.

- b. *Levispirillum peregrinum* subsp. *integrum* subsp. nov. (*Spirillum peregrinum* subsp. *integrum* Terasaki 1973, 60.) *in te.grum*. L. neut. adj. *integrum* unchanged (referring here to failure to form coccoid bodies).

Morphology and characteristics as for the species. Differs from the subsp. *peregrinum* by failing to form coccoid bodies as a predominant form even after 28 d of incubation.

The mol% G + C of the DNA is: 64 (T_m).

Type strain: MF 19, ATCC 33334, DSM 11589, IFO 13617.

Genus IV. *Magnetospirillum* Schleifer, Schöler and Ludwig 1992, 291^{VP} (Effective publication: Schleifer, Schöler and Ludwig in Schleifer, Schöler, Spring, Weizenegger, Amann, Ludwig and Köhler 1991, 384)

DIRK SCHÜLER AND KARL-HEINZ SCHLEIFER

Mag.ne' to.spi.ril' lum. Gr. n. *magnes* magnet, comb. form *magneto*–; Gr. n. *spira* a spiral; M.L. dim. neut. n. *spirillum* a small spiral; *Magnetospirillum* a small magnetic spiral.

Helical (clockwise) **spirilla**, $0.2\text{--}0.7 \times 3\text{--}4\ \mu\text{m}$. Occur in freshwater sediments. Gram negative. Motile by means of a single flagellum at each pole. Cells contain variable numbers of magnetosomes. Intracytoplasmic membranes (magnetosome membrane) are present. Intracellular poly-hydroxy-alkanoate formation. **Magnetotactic. Microaerophilic**, having a respiratory type of metabolism with oxygen as terminal electron acceptor. **Chemooorganotrophic**. Optimum growth temperature is 30°C. No growth in the presence of 1% NaCl. Catalase and oxidase can be absent or present. Denitrify under microaerobic conditions. Growth occurs on various organic acids; carbohydrates are not used. Members form a monophyletic group within the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 63–64.

Type species: *Magnetospirillum gryphiswaldense* Schleifer, Schöler and Ludwig 1992, 291 (Effective publication: Schleifer, Schöler and Ludwig in Schleifer, Schöler, Spring, Weizenegger, Amann, Ludwig and Köhler 1991, 384.)

FURTHER DESCRIPTIVE INFORMATION

Cells of *Magnetospirillum* are helical and $0.2\text{--}0.7 \times 3\text{--}4\ \mu\text{m}$. Enlarged pleomorphic forms and coccoid bodies are formed in older cultures and under conditions of excess oxygen. Each magnetotactic cell contains a number of electron-dense intracytoplasmic inclusion bodies (magnetosomes), which are aligned in a linear chain (for details of morphology and ultrastructure of the type strain *M. gryphiswaldense* see Fig. BXII.α.13). The magnetosomes consist of membrane-enveloped cubo-octahedral particles of magnetite (Fe_3O_4) (Balkwill et al., 1980). The size of mature magnetite crystals is 42 nm in diameter. The number of magnetosome particles per cell varies between 0 and 60 depending on growth conditions, oxygen, and iron supply. Magnetite formation requires low oxygen concentrations (<5 kPa) (Blakemore et al., 1985; Schöler and Baeuerlein, 1998). Concentrated suspensions of magnetic cells are dark gray-brown in color, those of nonmagnetic cells are light cream colored. Magnetite formation is coupled to the uptake of bulk amounts of ferric iron at high rates and cells intracellularly accumulate more than 2% iron (dry wt) during magnetite synthesis (Schöler and Baeuerlein, 1998). Production of a hydroxamate-type siderophore has been reported for *M. magnetotacticum* (Paoletti and Blakemore, 1986), but siderophore production could be not detected in *M. gryphiswaldense* (Schöler and Baeuerlein, 1996). Magnetite crystals are enveloped by the magnetosome membrane, which represents an intracytoplasmic membrane compartment

with a distinctive protein and lipid composition (Schöler and Baeuerlein, 1997; Gorby et al., 1988). Cells grown under low-iron conditions contain empty magnetosome vesicles. The magnetosome chain is responsible for the tactic response of the cells to magnetic fields that is assumed to have a navigational function by interaction with the Earth's magnetic field (Blakemore and Frankel, 1981). *Magnetospirilla* exhibit an axial type of magnetotaxis, that is, cells are capable of swimming parallel or antiparallel to the magnetic field by alternately reversing their swimming direction without turning (Frankel et al., 1997).

Magnetospirillum strains are obligately microaerophilic. The level of oxygen tolerance differs between species, and is apparently correlated to the presence or absence of the oxygen-protective enzyme catalase. In semi-solid medium incubated under air atmospheres, initial growth occurs as a thin band some distance from the surface. Colony formation on agar plates is difficult to achieve; surface colonies are formed in *M. magnetotacticum* if catalase is added to the medium (Blakemore et al., 1979) and in *M. gryphiswaldense* on ACA medium (Schultheiss and Schöler, 2003). Best growth occurs in simple media containing short organic acids and minerals (see Tables BXII.α.7 and BXII.α.8). A cytochrome *a*₁-like hemoprotein (Tamegai et al., 1993), and a cytochrome *c*₅₅₀ (Yoshimatsu et al., 1995) are present in *M. magnetotacticum*. A *ccb*-type cytochrome *c* oxidase functions as terminal oxidase in the microaerobic respiration of *M. magnetotacticum* (Tamegai and Fukumori, 1994). A cytochrome *cd*₁-type nitrite reductase with Fe(II):nitrite oxidoreductase activity and a speculated function in magnetite synthesis is present in *M. magnetotacticum* (Yamazaki et al., 1995). The purification of a ferric iron reductase that is loosely bound to the cytoplasmic membrane was described for *M. magnetotacticum* (Noguchi et al., 1999).

No growth factors are required. Growth occurs with fumarate, tartrate, malate, succinate, lactate, pyruvate, oxalacetate, malonate, β-hydroxybutyrate, and maleate as sole carbon sources. Carbohydrates are not used. N₂, nitrate, and ammonium can serve as sole nitrogen sources (Bazyliński and Blakemore, 1983b; Bazyliński et al., 2000). No growth under strictly anaerobic conditions. However, at low oxygen levels nitrate is dissimilated to nitrous oxide and nitrogen gas in *M. magnetotacticum* (Escalante-Semerena et al., 1980; Bazyliński and Blakemore, 1983a). *Magnetospirillum* species contain C_{18:1}, C_{16:1}, and C_{16:0} as the major non-polar fatty acids, and C_{14:0}, C_{16:0}, and C_{18:0} as 3-hydroxy acids; Q-10 is the major ubiquinone (Sakane and Yokota, 1994). *M. gryphiswaldense* and *M. magnetotacticum* contain putrescine and

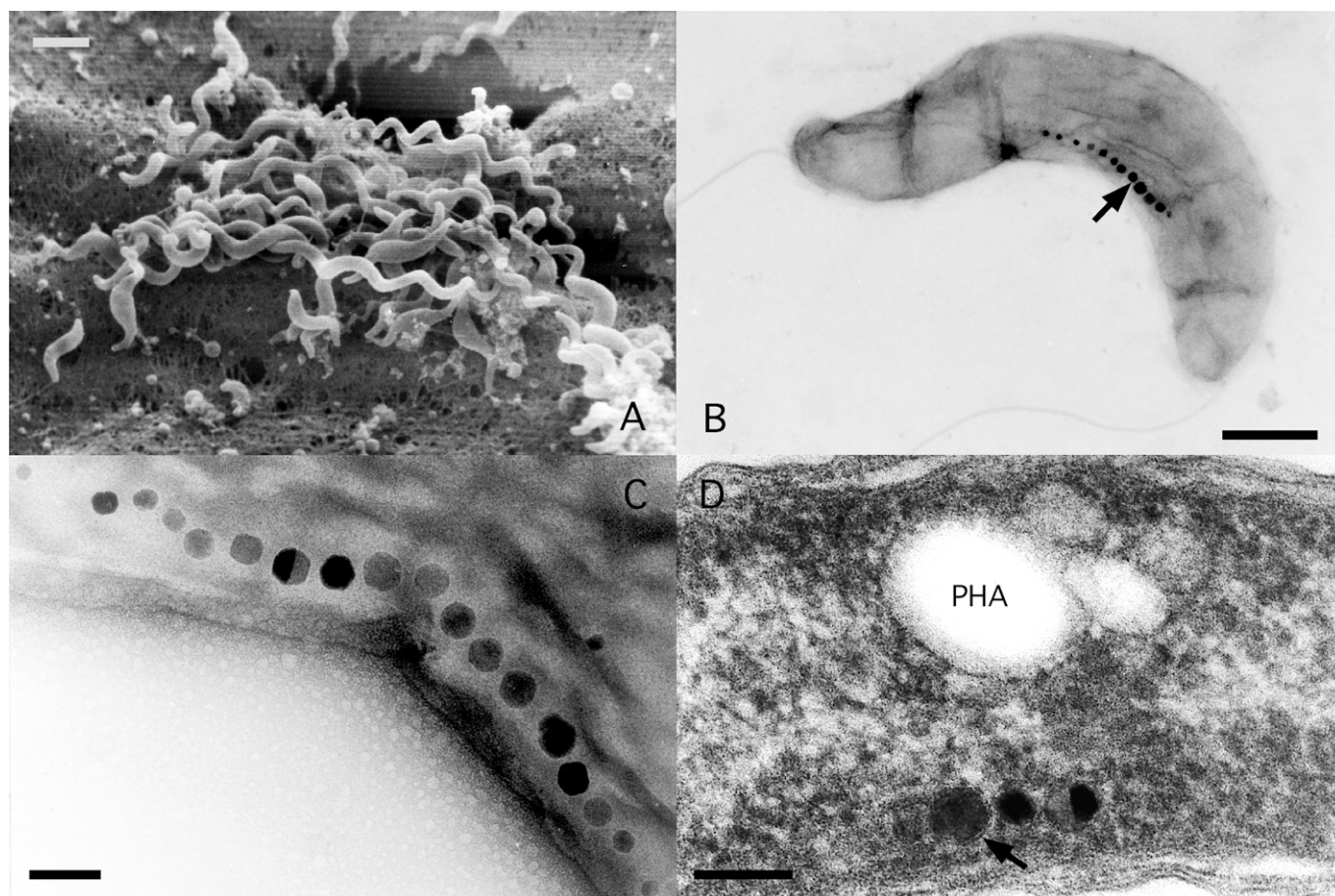


FIGURE BXII.α.13. (A) Scanning electron micrograph of cells of *M. gryphiswaldense* showing the characteristic helical morphology of *Magnetospirillum* species (Bar = 1 μ m). (B) Transmission electron micrograph of a negatively stained cell of *Magnetospirillum gryphiswaldense* showing the bipolar-monotrichous flagellation and the chain of magnetosomes (arrow) (Bar = 0.5 μ m). (C) Transmission electron micrograph of the magnetosome chain of *M. gryphiswaldense*. Cells contain intracellularly up to 60 cubo-octahedral crystals of magnetite (Fe_3O_4) that are 42 nm in size (Bar = 0.1 μ m). (D) Transmission electron microscopy of a thin-section of a magnetic cell of *M. gryphiswaldense*. Individual magnetite crystals are enveloped by the magnetosome membrane (arrow). Cells contain poly-hydroxy-alkanoate granules (PHA) (Bar = 0.1 μ m).

spermidine, or putrescine, cadaverine, and spermidine, respectively, as the major polyamines (Hamana et al., 1994).

ENRICHMENT AND ISOLATION PROCEDURES

Magnetospirillum species can be isolated from the oxic–anoxic transition zone of many freshwater sediments. Enrichments of magnetic spirilla can be obtained by the undisturbed incubation of loosely covered jars, filled with 1/3 mud and 2/3 water from the sample origin, during several months at room temperature. As no selective media for the cultivation of magnetic spirilla are

known, the effective physical separation of magnetotactic cells from contaminants is crucial in their isolation. Advantage can be taken of the directed, active migration of magnetospirilla in magnetic fields. Large numbers of magnetotactic bacteria can be collected from the sediment–water interface of the enrichments using a bar magnet. Magnetotactic bacteria can then be further separated by a “racetrack” method using semisolid agar or sterile capillaries (Wolfe et al., 1987; Schüller and Köhler, 1992). By application of a magnetic field, magnetotactic bacteria will out-swim contaminating organisms and a highly purified inoculum can be collected at the end of the capillary close to the south pole of a bar magnet. The purified inoculum is then transferred to prereduced semisolid, low-nutrient isolation medium, which is incubated under microaerobic atmospheres containing 0.6–1% oxygen (Blakemore et al., 1979). Alternatively, a two-layer sulfide–oxygen gradient medium has proven useful in the isolation of microaerophilic magnetospirilla by creation of a vertical redox gradient (Schüller et al., 1999).

MAINTENANCE PROCEDURES

Magnetospirilla may be maintained in semisolid growth medium at 30°C with weekly transfer or at room temperature with monthly transfer. Long-term preservation is accomplished by suspending a dense suspension of cells in liquid growth medium containing

TABLE BXII.α.7. Characteristics differentiating *Magnetospirillum gryphiswaldense* and *M. magnetotacticum*^a

Characteristics	<i>M. gryphiswaldense</i>	<i>M. magnetotacticum</i>
Catalase	+	–
Oxidase	+	– ^b
Growth and magnetosome formation in liquid culture exposed to air	+	–

^aFor symbols see standard definitions.

^bOxidase test is faintly positive with toluene-treated cells (Maratea and Blakemore, 1981).

TABLE BXII.α.8. Other characteristics of *M. gryphiswaldense* and *M. magnetotacticum*^a

Characteristics	<i>M. gryphiswaldense</i>	<i>M. magnetotacticum</i>
Cell size, µm	0.2–0.7 × 3–4	0.2–0.7 × 3–4
Magnetosomes: 0–60 intracellular cubo-octahedral magnetite (Fe ₃ O ₄) crystals, 42 nm	+	+
Magnetic reaction, sensitivity	+	+
Single bipolar flagella	+	+
Intracellular PHA-formation	+	+
Optimal growth temperature, °C	30	30
Growth in the presence of 1% NaCl	–	–
Growth on carbohydrates	–	–
Growth on fumarate, tartrate, malate, succinate, lactate, pyruvate, oxalacetate, malonate, β-hydroxybutyrate, maleate	+	+
Microaerobic reduction of nitrate	+	+
Growth with N ₂ as sole nitrogen source, nitrogenase activity	+	+
Hydrolysis of casein, starch, hippurate, esculin, gelatin	–	–
Hydrogen sulfide production from cysteine	–	–
Growth in the presence of 1% glycine	nd	+
Growth in the presence of 1% bile	nd	–
Anaerobic growth with NO ₃ [–] , Fe(III), DMSO, SO ₄ ^{2–b}	–	–
Formation of coccoid bodies in older cultures	+	+
Selenite reduction	nd	–
Pigment formation from aromatic amino acids	–	–
Urease, sulfatase, indole	nd	–
Alkaline reaction in litmus milk	nd	–

^aFor symbols see standard definitions; nd, not determined.^bVery slow ferric iron-dependent growth in the absence of oxygen has been reported (Guerin and Blakemore, 1992).

10% (v/v) dimethyl sulfoxide, with subsequent freezing in liquid nitrogen and storage at –80°C.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

The most conspicuous trait of *Magnetospirillum* species is their magnetic sensitivity, which can be easily checked in living cells by phase-contrast microscopy in the presence of a permanent magnet, where magnetospirilla will orient and swim parallel to the magnetic field lines. Magnetic reaction of cultures can also be checked by placing a drop of cell suspension onto the top of a magnetic stirrer and watching for the characteristic “flickering”, as the light scattering of suspensions of magnetic cells is affected by changing magnetic fields (Schüler et al., 1995). By electron microscopy, intracellular magnetosome crystals are easily revealed in unstained cells as electron-dense inclusions.

A *Magnetospirillum*-specific pair of primers for the PCR-amplification of a 16S rRNA gene fragment has been proposed (Burgess et al., 1993), but these were found to also be homologous to several unrelated, nonmagnetic *Alphaproteobacteria* in a recent database search (D. Schüler, unpublished). Tests for oxygen tolerance and catalase and oxidase activities are further useful diagnostic features.

DIFFERENTIATION OF THE GENUS *MAGNETOSPIRILLUM* FROM OTHER GENERA

The intracellular magnetosome formation and magnetic reaction clearly distinguishes *Magnetospirillum* from other microaerophilic, spiral-shaped bacteria from freshwater.

Magnetospirillum species are most closely related (94–96% 16S rDNA sequence similarity) to several nonmagnetic, photosynthetic, nonsulfur purple *Alphaproteobacteria* of the genus *Phaeospirillum* (*P. fulvum* and *P. molischianum*; Imhoff et al., 1998), with which they share similarities in cell morphology and flagellation, the presence of intracytoplasmic membranes, and the microaerophilic nature of their chemoorganotrophic growth. However, *Magnetospirillum* strains are unable to grow photosynthetically and lack the characteristic photosynthetic pigments (bacteriochlorophyll, carotenoids) of the *Phaeospirillum* species.

Magnetospirillum species are phylogenetically only distantly related to other, mostly uncultured vibrioid, rod-shaped, and coccoid magnetotactic bacteria, and can be distinguished by cell morphology and the shape, size, and arrangement of the magnetosome crystals (Spring and Schleifer, 1995).

TAXONOMIC COMMENTS

The first magnetic spirillum isolated by R. Blakemore was originally described as *Aquaspirillum magnetotacticum* (Maratea and Blakemore, 1981). However, 16S rRNA gene sequence analysis of *A. magnetotacticum* and the newly isolated magnetic strain MSR-1 demonstrated their affiliation with the *Alphaproteobacteria*, while the type species of *Aquaspirillum* falls in the *Betaproteobacteria*. Moreover, magnetic spirilla differ from known *Aquaspirillum* strains in a number of morphological and physiological features such as the presence of magnetic inclusions and the type of flagellation, suggesting classification in a separate genus. Therefore, the new genus *Magnetospirillum* was proposed with the type

strain *M. gryphiswaldense* MSR-1, and *A. magnetotacticum* was transferred to *Magnetospirillum* as *M. magnetotacticum* (see Tables BXII.α.7 and BXII.α.8) (Schleifer et al., 1991).

FURTHER READING

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Schleifer, K.H., D. Schüller, S. Spring, M. Weizenegger, R. Amann, W.

Lduwig and M. Köhler. 1991. The genus *Magnetospirillum*, gen. nov., description of *Magnetospirillum gryphiswaldense*, sp. nov. and transfer of *Aquaspirillum magnetotacticum* to *Magnetospirillum magnetotacticum*, comb. nov. *Syst. Appl. Microbiol.* 14: 379–385.

Schüler, D. 1999. Formation of magnetosomes in magnetotactic bacteria. *J. Mol. Microbiol. Biotechnol.* 1: 79–86.

Spring, S. and K.H. Schleifer. 1995. Diversity of magnetotactic bacteria. *Syst. Appl. Microbiol.* 18: 147–153.

List of species of the genus *Magnetospirillum*

1. ***Magnetospirillum gryphiswaldense*** Schleifer, Schüller and Ludwig 1992, 291^{VP} (Effective publication: Schleifer, Schüller and Ludwig in Schleifer, Schüller, Spring, Weizenegger, Amann, Ludwig and Köhler 1991, 384.)
gry.phis.wal.den' se. L. adj. *gryphiswaldense* the Latin name of Greifswald, a town in Germany where the organism was isolated.

Helical spirilla, $0.7 \times 3\text{--}4\text{ }\mu\text{m}$. Catalase and oxidase positive. Microaerophilic, but grows prolifically in agitated liquid medium¹ exposed to air if large inocula (1/10) are used. Growth rates are $0.3\text{--}0.1\text{ h}^{-1}$. Isolated by D. Schüller from sediments of a small river (Ryck) near Greifswald, Germany.

The mol% G + C of the DNA is: 62.7 (HPLC) (Sakane and Yokota, 1994).

Type strain: MSR-1, DSM 6361.

GenBank accession number (16S rRNA): Y10109.

2. ***Magnetospirillum magnetotacticum*** (Maratea and Blakemore 1981) Schleifer, Schüller and Ludwig 1992, 291^{VP} (Effective publication: Schleifer, Schüller and Ludwig in Schleifer, Schüller, Spring, Weizenegger, Amann, Ludwig and Köhler 1991, 385) (*Aquaspirillum magnetotacticum* Maratea and Blakemore 1981, 454.)

mag.ne.to.tac.'ti.cum. Gr. n. *magnes* magnet, comb. form *mag-neto*- Gr. adj. *taktikos* showing orientation or movement di-

rected by a force or agent; *magnetotacticum* capable of orientation with respect to a magnet.

Helical spirilla, $0.4\text{--}0.7 \times 3\text{--}4\text{ }\mu\text{m}$. Microaerophilic. No growth of magnetic cells in liquid cultures with free gas exchange to air. Very weak ferric iron-dependent growth in the absence of oxygen has been reported (Guerin and Blakemore, 1992). Nitrate is reduced to N_2 with transient accumulation of nitrous oxide but without nitrite accumulation (Bazylinski and Blakemore, 1983b). Grows in a defined mineral medium². Vitamins are not strictly required for growth, but deletion of vitamins from the growth medium results in a pleomorphic appearance (Blakemore et al., 1979). Catalase, oxidase, urease, sulfatase, and indole are negative. Oxidase test is faintly positive with toluene-treated cells (Maratea and Blakemore, 1981). Isolated by R.P. Blakemore at the University of Illinois from sediments collected in Cedar Swamp, Woods Hole, Massachusetts (USA).

The mol% G + C of the DNA is: 63 (HPLC) (Sakane and Yokota, 1994).

Type strain: MS-1, ATCC 31 632, DSM 3856.

GenBank accession number (16S rRNA): Y10110.

Other Organisms

Several magnetic spirilla isolates have been identified as *Magnetospirillum* species based on cell morphology, ultrastructure, and phylogenetic analysis, and form a monophyletic group including *M. magnetotacticum* and *M. gryphiswaldense* within the *Alphaproteobacteria*. However, their taxonomic characterization is incomplete and they are not yet validly described.

The *Magnetospirillum* strains MGT-1 (GenBank accession number of the 16S rRNA gene sequence: D17515) and AMB-1 (D17514) were isolated from freshwater ponds in Tokyo, Japan (Burgess et al., 1993). Sequence similarities of the 16S rRNA genes of MGT-1 and AMB-1 to that of *M. gryphiswaldense* are 95.0% and 95.8%, respectively, and to that of *M. magnetotacticum* are 99.0% and 99.4% (Schüller et al., 1999). MGT-1 and AMB-1 were reported to have increased oxygen tolerance and to form colonies on agar surfaces exposed to air (Burgess et al., 1993). Seven *Magnetospirillum* strains (MSM-1, 3, 4, 6, 7, 8, 9) were iso-

lated from a freshwater pond in Iowa, USA (EMBL-accession numbers for the sequences of the 16S rRNA genes of the *Magnetospirillum* strains MSM-3, MSM-4, and MSM-6 are Y17389, Y17390, and Y17391, respectively). While five of the isolates were very similar to either *M. gryphiswaldense* or *M. magnetotacticum* by 16S rRNA gene sequence analysis (>99.7%), two new isolates (MSM-3, MSM-4) are likely to represent new species (Schüller et al., 1999). A total of six different sequences was obtained from a single sample site, indicating a significant microdiversity of natural populations of magnetic spirilla.

1. *M. gryphiswaldense* medium (mod. after Schüller and Baeuerlein, 1996) contains per liter: KH_2PO_4 , 0.5 g; Na-acetate, 1.0 g; soybean peptone (E. Merck), 1.0 g; NH_4Cl , 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; yeast extract, 0.1 g; agar (for semisolid medium), 2 g; Fe(III) citrate, 50 μM (final concentration). Dissolve and autoclave at 121°C for 15 min. For liquid cultures, inoculate with 1/10 vol and agitate at 100 rpm.

2. *M. magnetotacticum* medium (DSM 380) contains per liter: Vitamin solution (Wolin et al., 1963), 10 ml; trace elements (Wolin et al., 1963), 5 ml; Fe(III) quinate solution, 2 ml; resazurine, 0.5 mg; KH_2PO_4 , 0.68 g; NaNO_3 , 0.12 g; Na-thioglycolate, 0.05 g; L(+)-tartaric acid, 0.3 g; succinic acid, 0.37 g; Na-acetate, 0.05 g; agar (for solid medium), 13.0 g. Dissolve components in the order given, adjust pH to 6.75 with NaOH. Ferric quinate solution, 0.01 M (per liter): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 4.5 g; quinic acid, 1.9 g. Dissolve and autoclave at 121°C for 15 min. Liquid medium: Purge medium with N_2 gas for 10 min. Under the same atmosphere, anaerobically fill tubes to 1/3 of their volume and seal. Autoclave at 121°C for 15 min. Before inoculation, add sterile air (with hypodermic syringe through the rubber closure) to 1% O_2 concentration in the gas phase.

Genus V. *Phaeospirillum* Imhoff, Petri, and Siling 1998, 796^{VP}

JOHANNES F. IMHOFF

Phae.o.spi.ril' lum. Gr. adj. *phaeos* brown; M.L. neut. n. *Spirillum* a bacterial genus; M.L. neut. n. *Phaeospirillum* brown *Spirillum*.

Cells are vibrioid to spiral shaped, motile by means of polar flagella and multiply by binary fission. **Gram negative and belong to the Alphaproteobacteria.** Internal photosynthetic membranes are lamellar stacks forming a sharp angle with the cytoplasmic membrane. Photosynthetic pigments are **bacteriochlorophyll a** (esterified with phytol) and **carotenoids of the spirilloxanthin series**, with spirilloxanthin itself lacking. **Ubiquinones and menaquinones with nine isoprene units (Q-9 and MK-9)** are present. **Major cellular fatty acids are C_{18:1}, C_{16:1}, and C_{16:0}**, with C_{18:1} as dominant component (44–55% of total fatty acids).

Cells grow preferentially photoheterotrophically under anoxic conditions in the light. **Chemotrophic growth is possible at very low oxygen tensions in the dark.** Growth factors may be required. **Mesophilic freshwater bacteria with preference for neutral pH.** Habitat: stagnant and anoxic freshwater habitats.

The mol% G + C of the DNA is: 60.5–65.3.

Type species: *Phaeospirillum fulvum* (van Niel 1944) Imhoff, Petri, and Siling 1998, 797 (*Rhodospirillum fulvum* van Niel 1944, 108.)

FURTHER DESCRIPTIVE INFORMATION

Phaeospirillum species are very sensitive to oxygen and do not grow under oxic conditions in the dark (Pfennig, 1969b). They do grow, however, under microoxic conditions in the dark, provided the oxygen tension is lower than 1.5 kPa for *Phaeospirillum fulvum* and 1.0 kPa for *Phaeospirillum molischianum* (Lehmann, 1976). These bacteria are unable to induce a second electron transport chain in the presence of oxygen and depend on microoxic conditions in which the internal membrane system and the light-driven electron transport chain are fully expressed. The cells are fully pigmented under these growth conditions (Lehmann, 1976).

Ammonia assimilation is mediated by the glutamine synthetase/glutamate synthase reactions, which are NADH-dependent in *Phaeospirillum molischianum* and *Phaeospirillum fulvum* (Brown and Herbert, 1977).

ENRICHMENT AND ISOLATION PROCEDURES

Media and growth conditions applied for *Rhodospirillum* species are also suitable for *Phaeospirillum* species, if the oxygen partial pressure is adequately reduced. This can be achieved by addition of 0.05% sodium ascorbate to the growth media in completely

filled screw-capped bottles. A recipe for a mineral medium appropriate for cultivation of *Phaeospirillum* species is given with the description of the genus *Rhodospirillum*. Higher fatty acids such as pelargonate and caprylate (concentrations below 0.04% at pH 7.5) provide selective conditions for enrichment of *P. fulvum* and *P. molischianum*. Benzoate may be used for selective enrichment of *P. fulvum*. Standard techniques for isolation of anaerobic bacteria in agar dilution series and on agar plates can be applied to *Phaeospirillum* species (Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Truper, 1992), if care is taken to establish and maintain oxygen-free conditions.

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen or at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *PHAEOSPIRILLUM* FROM OTHER GENERA

Besides 16S rDNA sequence similarities (Kawasaki et al., 1993b; Imhoff et al., 1998), the size and sequences of the small-size cytochromes *c*₂ (Ambler et al., 1979), quinone, and fatty acid composition and other properties also distinguish *Phaeospirillum* and *Rhodospirillum* species (see Tables BXII..1 and BXII..2 in the chapter describing the genus *Rhodospirillum*).

TAXONOMIC COMMENTS

Phaeospirillum species have previously been included in the genus *Rhodospirillum*. Based on significant differences in chemotaxonomic properties and 16S rDNA sequences, they have been transferred into the new genus *Phaeospirillum* (Imhoff et al., 1998). The name *Phaeospirillum* was first proposed by Kluyver and van Niel (1936) for brown-colored, phototrophic, spiral-shaped bacteria but was not included in the Approved List of Names and therefore lost standing in nomenclature. The name was revived (Imhoff et al., 1998). Both *Phaeospirillum* species have highly similar 16S rDNA sequences (greater than 99% similarity) that would allow their treatment as a single species. This was not proposed because of the well-recognized phenotypic differences between the two species. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *PHAEOSPIRILLUM*

Phaeospirillum molischianum and *Phaeospirillum fulvum* can be distinguished from each other primarily by cell size, growth factor requirements, and fatty acid composition (Table BXII..1 in the description of the genus *Rhodospirillum*). The utilization of ben-

zoate is a characteristic and distinguishing property of *Phaeospirillum fulvum* (Table BXII..2 in the description of the genus *Rhodospirillum*).

List of species of the genus *Phaeospirillum*

1. ***Phaeospirillum fulvum*** (van Niel 1944) Imhoff, Petri, and Siling 1998, 797^{VP} (*Rhodospirillum fulvum* van Niel 1944, 108.)
ful'vum. M.L. neut. adj. *fulvum* deep or reddish yellow, tawny.

Cells are vibrioid shaped to spiral, 0.5–0.7 × 3.5 µm; one complete turn of a spiral is 1.0–1.6 µm wide. Internal photosynthetic membranes are present as lamellar stacks forming a sharp angle to the cytoplasmic membrane. Anaerobic liquid cultures are deep brown. Photosynthetic pig-

ments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spirilloxanthin series. Spirilloxanthin is lacking, but the biosynthetic precursors lycopene and rhodopin are present as major components.

Cells grow preferentially photoheterotrophically under anoxic conditions in the light with various organic compounds as carbon and electron sources. Chemotrophic growth at very low oxygen tensions is possible. Unable to adapt to oxic growth conditions. Under microoxic conditions cells are fully pigmented. Carbon sources utilized are shown in Table BXII.α.2 in the description of the genus *Rhodospirillum*. Ammonia and N₂ are used as nitrogen source. Sulfate can be used as sole sulfur source. For optimal development, the addition of ascorbate or thioglycolate as a reductant may be necessary. *p*-Aminobenzoic acid is required as a growth factor.

Mesophilic freshwater bacterium with optimum growth at 25–30°C and pH 7.3 (pH range: 6.0–8.5). Habitat: stagnant and anoxic freshwater habitats that are exposed to the light.

The mol% G + C of the DNA is: 64.3–65.3 (Bd) and 62.1–62.8 (*T_m*).

Type strain: Pfennig 1360, ATCC 15798, DSM 113.

GenBank accession number (16S rRNA): M14433, M59065.

2. ***Phaeospirillum molischianum*** (Giesberger 1947) Imhoff, Petri, and Söling 1998, 797^{VP} (*Rhodospirillum molischianum* Giesberger 1947, 142.)

mo.li'schi.a' num. M.L. neut. adj. *molischianum* pertaining to Molisch, named for H. Molisch, an Austrian botanist.

Cells are vibrioid to spiral shaped, 0.7–1.0 × 4–6 µm or even longer; one complete turn of a spiral is 1.5–2.5 µm wide. Internal photosynthetic membranes are present as lamellar stacks forming a sharp angle to the cytoplasmic membrane (Fig. BXII.α.14). Anaerobic liquid cultures are brown-orange to brown-red or dark brown. Absorption maxima of living cells are at 375, 465, 488–491, 520–528, 590–595, 803–807, and 850–855 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spirilloxanthin series. Spirilloxanthin is lacking, but the biosynthetic precursors lycopene and rhodopin are present as major components. Cells grow preferentially photoheterotrophically under anoxic conditions in the light with various organic compounds as carbon and electron sources. Chemotrophic growth at very low oxygen tensions is possible, but the ability to adapt to oxic growth conditions is lacking. Under microoxic conditions cells are fully pigmented. Carbon sources utilized are shown in Table BXII.α.2 in the description of the genus *Rhodospirillum*. Malonate is not used. Ammonia, N₂, and some amino acids are used as nitrogen sources. For optimal development the addition of ascorbate or thioglycolate as a reductant may be necessary. Complex mixtures of amino acids, such as yeast extract and vitamin-free Casamino acids, stimulate growth considerably and may be required.

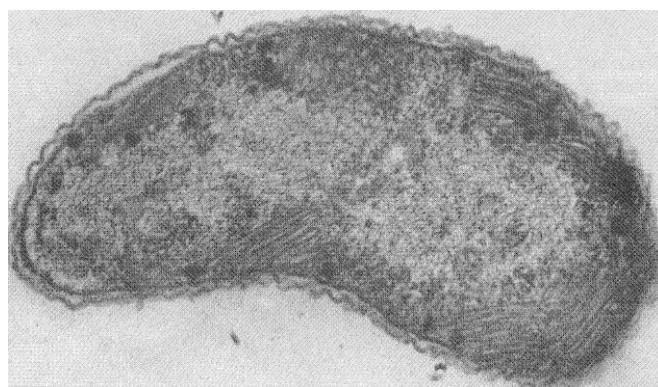


FIGURE BXII.α.14. *Phaeospirillum molischianum* grown anaerobically in the light. Note the position and the lamellar stack type of the intracytoplasmic membrane system. × 90,000. (Courtesy of G. Drews.)

Mesophilic freshwater bacterium with optimal growth at 30°C and pH 7.3 (pH range: 6.0–8.5). Habitat: stagnant and anoxic freshwater habitats that are exposed to the light.

The mol% G + C of the DNA is: 60.5–64.8 (Bd) and 62.1–62.6 (*T_m*).

Type strain: ATCC 14031, DSM 120, NCIB 9957, NTHC 131.

GenBank accession number (16S rRNA): M59067.

Genus VI. *Rhodocista* Kawasaki, Hoshino, Kuraishi and Yamasato 1994b, 182^{VP} (Effective publication: Kawasaki, Hoshino, Kuraishi and Yamasato 1992, 548)

JOHANNES F. IMHOFF

Rho.do.ci' sta. L. fem. n. *rhodon* the rose; L. fem. n. *cista* a basket; M.L. fem. n. *Rhodocista* the rose basket.

Cells are vibrioid to spiral in shape, motile by means of a long polar flagellum. Gram negative. Belong to the *Alphaproteobacteria*. Internal photosynthetic membranes occur as lamellae lying parallel to the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Ubiquinone with nine isoprene units (Q-9) is present.

Cells grow preferentially photoheterotrophically under anoxic conditions in the light. Chemotrophic growth under oxic conditions in the dark is possible.

The mol% G + C of the DNA is: 69.9.

Type species: ***Rhodocista centenaria*** Kawasaki, Hoshino, Kuraishi and Yamasato 1994b, 182 (Effective publication: Kawasaki,

Hoshino, Kuraishi and Yamasato 1992, 548) (*Rhodospirillum centenarium* Favinger, Stadtwald and Gest 1994, 182.)

FURTHER DESCRIPTIVE INFORMATION

When grown in liquid culture, *R. centenaria* cells are motile by means of a single polar flagellum (Favinger et al., 1989). On solid media, however, numerous lateral, peritrichous flagella that enable rapid movement on agar surfaces are produced. The kind of swarming motility observed with *R. centenaria* has not yet been observed in other phototrophic purple nonsulfur bacteria, and enables very rapid light-directed motility across the agar surface, which appears to be a true phototactic behavior (Ragatz et al.,

1995). It has also been shown that photo-induced cyclic electron flow is required for the photo-tactical response and that phototaxis is controlled by respiration (Romagnoli et al., 1997). The type strain (ATCC 43720) is not phototactically active (Nickens et al., 1996).

Absorption spectra of cultures grown anaerobically in the light or aerobically in the dark are almost identical, which demonstrates the absence of oxygen repression of photopigment synthesis that is common to most of the anoxygenic phototrophic purple bacteria (Yildiz et al., 1991).

Out of 13 strains under investigation, all were sensitive to rifampicin and streptomycin but highly resistant to neomycin and kanamycin (Nickens et al., 1996).

ENRICHMENT AND ISOLATION PROCEDURES

R. centenaria appears to be widely distributed in warm waters and has been repeatedly isolated from a variety of hot springs with the source water temperature between 34 and 58°C (Nickens et al., 1996). Most of the strains have been isolated with dinitrogen as sole nitrogen source and under anoxic conditions, but after transient exposure to air. Incubation at temperatures between 40 and 45°C under anoxic conditions in the light, in media suitable for freshwater purple nonsulfur bacteria, and with N₂ as the sole nitrogen source, provides selective conditions for the enrichment of *Rhodocista centenaria* (Nickens et al., 1996). Standard techniques for the isolation of anaerobic bacteria in agar dilution series and on agar plates can be applied (Biebl and Pfennig, 1981; Imhoff and Trüper, 1992).

MAINTENANCE PROCEDURES

Cultures are well preserved by standard techniques in liquid nitrogen, by lyophilization or storage at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOCISTA* FROM OTHER GENERA

Differential characteristics and phylogenetic relationships based on 16S rDNA sequences are shown in Table BXII.α.1 and Table BXII.α.2 in the description of the genus *Rhodospirillum* and Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

TAXONOMIC COMMENTS

Until recently, the genus *Rhodospirillum* represented a heterogeneous assemblage of species that were of spiral shape and capable of phototrophic growth. Four halophilic species were classified together with several freshwater species. Because of its spiral shape and phototrophic capacity, *Rhodospirillum centenarium* was also assigned to the genus *Rhodospirillum* (Favinger et al., 1989). The anticipated heterogeneity of the genus *Rhodospirillum* became clearly apparent with the 16S rDNA sequence information of most of the known species (Kawasaki et al., 1993b), and these data implied that the spiral-shaped *Alphaproteobacteria* were phylogenetically quite distantly related to each other and did not warrant classification in one and the same genus. Upon recognition of the great genetic distance between *Rhodospirillum rubrum* and *Rhodospirillum centenarium*, the latter was transferred to a new genus as *Rhodocista centenaria* (Kawasaki et al., 1992). The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

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- Imhoff, J.F., R. Petri and J. Süling. 1998. Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the α -*Proteobacteria*: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomense* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *Int. J. Syst. Bacteriol.* 48: 793-798.
- Kawasaki, H., Y. Hoshino, H. Kuraishi and K. Yamasato. 1992. *Rhodocista centenaria* gen. nov., sp. nov., a cyst-forming anoxygenic photosynthetic bacterium and its phylogenetic position in the *Proteobacteria* alpha group. *J. Gen. Appl. Microbiol.* 38: 541-551.
- Kawasaki, H., Y. Hoshino and K. Yamasato. 1993. Phylogenetic diversity of phototrophic purple non-sulfur bacteria in the *alpha proteobacteria* group. *FEMS Microbiol. Lett.* 112: 61-66.

List of species of the genus *Rhodocista*

1. ***Rhodocista centenaria*** Kawasaki, Hoshino, Kuraishi and Yamasato 1994b, 182^{VP} (Effective publication: Kawasaki, Hoshino, Kuraishi and Yamasato 1992, 548) (*Rhodospirillum centenarium* Favinger, Stadtwald and Gest 1994, 182.) *cen.ten.a'r.i.a.* L. fem. adj. *centenaria* relating to a hundred, to commemorate a century after the publication of the first description of a phototrophic bacterium in 1887.

Cells are vibrioid to spiral in shape, 1-2 × 3 µm. In liquid media they are actively motile by means of a single polar flagellum. When grown on agar, peritrichous flagella are induced, conferring swarming motility. Swarming cells exhibit phototactic behavior. Cells are converted to thick-walled cysts, which are resistant to desiccation and heat, under conditions that also favor the formation of poly-β-hydroxybutyrate, especially in aging conditions on agar. Internal photosynthetic membranes are lamellae lying parallel to the cytoplasmic membrane. Color of anaerobically grown cultures is pink. Living cells show absorption maxima

at 475-550, 587, 800, and 875 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Photopigment synthesis is not significantly affected by molecular oxygen.

Cells grow preferentially photoheterotrophically under anoxic conditions in the light or chemotrophically under oxic conditions in the dark. Pyruvate, lactate, acetate, alanine, aspartate, glutamate, and glutamine are used as carbon sources and electron donors. Butyrate, valerate, caproate, and caprylate also support phototrophic growth when supplemented with bicarbonate (9 mM). Sugars and C4 dicarboxylic acids, including malate, do not support growth. With butyrate as the sole carbon source, the spiral-shaped cells are converted to cysts, which are desiccation and heat resistant. Filter-fixed cysts kept viability at 55°C for 2 d. Ammonia, N₂, glutamate, glutamine, aspartate, and alanine are utilized as nitrogen sources. Biotin and vitamin B₁₂ are required as growth factors.

Freshwater bacterium preferring elevated temperatures, with optimal growth at 40–45°C (upper limit 47°C) and pH 6.8 (pH range 5.7–7.0). Ubiquinone with nine isoprene units (Q-9) is the major quinone. Habitat: neutral to alkaline hot spring effluents, warm soils, effluents of paper factories.

The mol% G + C of the DNA is: 69.9 (T_m).
Type strain: ATCC 43720, DSM 9894, IAM 14193.
GenBank accession number (16S rRNA): D12701.

Genus VII. *Rhodospira* Pfennig, Lünsdorf, Süling and Imhoff 1998, 328^{VP} (Effective publication: Pfennig, Lünsdorf, Süling and Imhoff 1997, 44)

JOHANNES F. IMHOFF

Rho' do. spi' ra. Gr. n. *rhodon* the rose; Gr. n. *spira* the spiral; M.L. fem. n. *Rhodospira* the rose spiral.

Cells are vibrioid to spirilloid in shape, motile by means of flagella and multiply by binary fission. **Gram negative. Belong to the Alphaproteobacteria.** Internal photosynthetic membranes are of the vesicular type. Photosynthetic pigments are **bacteriochlorophyll *b*** and **carotenoids. Menaquinones and ubiquinones with seven isoprene units (MK-7 and Q-7)** are present. **Major cellular fatty acids are C_{18:1} and C_{16:0}**, with C_{18:1} as dominant component (~60% of total fatty acids).

Cells grow preferentially photoheterotrophically under anoxic conditions in the light, but poor growth may also be possible under microoxic conditions in the dark. Growth factors are required. **Marine bacteria** requiring salt for growth.

Habitat: salt marsh sediments and laminated microbial mats.

The mol% G + C of the DNA is: 65.7.

Type species: ***Rhodospira trueperi* Pfennig, Lünsdorf, Süling and Imhoff 1998, 328** (Effective publication: Pfennig, Lünsdorf, Süling and Imhoff 1997, 44.)

FURTHER DESCRIPTIVE INFORMATION

At present, *Rhodospira trueperi* is the only representative of this genus. *R. trueperi* was isolated from a flat laminated microbial mat in a salt marsh near Woods Hole, Massachusetts, USA. The spiral-shaped bacterium is highly motile, has bipolar tufts of flagella, and internal photosynthetic membranes of the vesicular type. Major photosynthetic pigments are bacteriochlorophyll *b* and the carotenoid tetrahydrospirilloxanthin. *R. trueperi*, a marine organism, shows optimum growth in the presence of salt. It utilizes a number of organic substrates as carbon and energy sources, and requires vitamins and sulfide as a reduced sulfur source for growth. In the presence of sulfide, S⁰ globules are formed outside the cells. S⁰ is not further oxidized to sulfate. *Rhodospira trueperi* has a unique lipid and fatty acid composition (Pfennig et al., 1997). The latter is dominated by C_{18:1} and C_{16:0} and is unique among the spiral-shaped phototrophic Alphaproteobacteria (Table BXII.α.1 in the description of the genus *Rhodo-*

spirillum). According to 16S rRNA gene sequence analysis, *Rhodospira trueperi* is most similar to *Roseospira mediosalina*. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

ENRICHMENT AND ISOLATION PROCEDURES

Rhodospira trueperi was isolated from a microbial mat in a salt marsh, requires salt for growth, and depends on the presence of sulfide in growth media (Pfennig et al., 1997). Standard techniques for isolation of anaerobic bacteria in agar dilution series can be applied for *Rhodospira* species (Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Trüper, 1992), if care is taken to establish and maintain oxygen-free conditions. *Rhodospira trueperi* can be grown on Pfennig's medium for purple sulfur bacteria (see genus *Chromatium*) with the addition of 2% NaCl and vitamins (or yeast extract).

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen or at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOSPIRA* FROM OTHER GENERA

Rhodospira species are characterized by salt requirements typical of marine bacteria, requirement for sulfide as a reduced sulfur source, unusual absorption spectra due to the presence of bacteriochlorophyll *b*, and presence of ubiquinones and menaquinones with seven isoprene units (see Table BXII.α.1). Major differentiating properties between *Rhodospira trueperi* and other phototrophic Alphaproteobacteria are shown in Table BXII.α.1 and carbon sources utilized in Table BXII.α.2 in the description of the genus *Rhodospirillum*. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

List of species of the genus Rhodospira

1. ***Rhodospira trueperi* Pfennig, Lünsdorf, Süling and Imhoff 1998, 328^{VP}** (Effective publication: Pfennig, Lünsdorf, Süling and Imhoff 1997, 44.)

true' pe. ri. M.L. gen. n. *trueperi* of Trüper, named for Hans Georg Trüper, a German microbiologist who contributed significantly to our knowledge of the anoxygenic phototrophic bacteria.

Cells vibrioid to spirilloid in shape, 0.6–0.8 × 1.5–3.0

µm, and motile by bipolar tufts of flagella (2–5 fibrils). Internal photosynthetic membranes are of the vesicular type. Anaerobically grown cultures are beige to peach-colored. Living cell suspensions show absorption maxima at 397, 458, 490, 600, 689, 801, 889, and 986 nm. Photosynthetic pigments are bacteriochlorophyll *b* and the carotenoid tetrahydrospirilloxanthin.

Grows photoheterotrophically under strictly anoxic con-

ditions in the light and in the presence of sulfide as a reduced sulfur source. In the presence of sulfide and hydrogen carbonate and under anoxic conditions, acetate, pyruvate, propionate, butyrate, valerate, lactate, fumarate, malate, succinate, and crotonate are used as substrates. Ethanol and cyclohexane carboxylate are not used. Weak growth may occur under microoxic conditions in the dark. Sulfide is oxidized to S^0 , which cannot be oxidized further to sulfate. Thiosulfate is not oxidized. Biotin, thiamine, and pantothenate are required as growth factors.

Marine bacterium with optimal growth at 25–30°C, pH 7.3–7.5 and 2% NaCl (in the presence of 0.3% $MgCl_2 \cdot 6H_2O$). Sodium chloride is required for growth, growth range is from 0.5–5.0% NaCl. Habitat: peach-colored layer of a laminated microbial mat of Great Sippewissett Salt Marsh.

The mol% G + C of the DNA is: 65.7 (by chemical analysis).

Type strain: Pfennig 8316, ATCC 700224.

GenBank accession number (16S rRNA): X99671.

Genus VIII. *Rhodovibrio* Imhoff, Petri and Süling 1998, 797^{VP}

JOHANNES F. IMHOFF

Rho.do.vi' bri.o. Gr. n. *rhodon* the rose; M.L. masc. n. *Vibrio* a bacterial genus; M.L. masc. n. *Rhodovibrio* the rose *Vibrio*.

Cells are vibrioid to spiral shaped, motile by means of polar flagella and multiply by binary fission. **Gram negative, belonging to the *Alphaproteobacteria***. Internal photosynthetic membranes are present as vesicles. Photosynthetic pigments are **bacteriochlorophyll *a*** and **carotenoids of the spirilloxanthin series**. **Ubiquinones and menaquinones with 10 isoprene units (Q-10 and MK-10) are present**. **Major cellular fatty acids are $C_{18:1}$ and $C_{18:0}$** .

Cells grow preferentially photoheterotrophically under anoxic conditions in the light. **Chemotrophic growth is possible under microoxic to oxic conditions in the dark**. Complex nutrients are required. **Halophilic bacteria that require NaCl or sea salt for growth** and have salt optima above seawater salinity. Habitat: anoxic zones of hypersaline environments such as salterns and salt lakes that are exposed to the light.

The mol% G + C of the DNA is: 66–67.

Type species: *Rhodovibrio salinarum* (Nissen and Dundas 1985) Imhoff, Petri and Süling 1998, 797 (*Rhodospirillum salinarum* Nissen and Dundas 1985, 224.)

FURTHER DESCRIPTIVE INFORMATION

Growth of *Rhodovibrio* species depends on hypersaline concentrations of salts. Both species grow well at 2 M NaCl. They show best growth in the presence of 0.1 M Mg^{2+} and can grow in media containing 1 M $MgCl_2$ (Mack et al., 1993). In contrast to *R. sodomensis*, which is tolerant to NaBr up to concentrations of 1.5 M, growth of *R. salinarum* is inhibited at much lower concentrations of NaBr (Mack et al., 1993). In response to the external salinity, glycine betaine and ectoine are accumulated as major and minor components, respectively, of the compatible solutes (Severin et al., 1992; Imhoff, 1992).

R. salinarum and *R. sodomensis* are unique among the spiral-shaped phototrophic *Alphaproteobacteria* in that they do not contain a soluble cytochrome c_2 (Moschetti et al., 1997; Bonora et al., 1998). *R. salinarum*, but not *R. sodomensis*, lacks a branched respiratory chain if grown aerobically in the dark (Moschetti et al., 1997). It contains two different high-potential iron–sulfur proteins (HiPIP), whereas *R. sodomensis* lacks HiPIPs (Bonora et al., 1998).

ENRICHMENT AND ISOLATION PROCEDURES

Rhodovibrio species are characterized by their requirement for high salt concentrations and complex nutrients. Therefore, complex media with salt concentrations of 10% and incubation anaerobically in the light provide selective conditions for the enrichment of *Rhodovibrio* species. Standard techniques for isolation

of anaerobic bacteria in agar dilution series and on agar plates can be applied for *Rhodovibrio* species (Imhoff, 1988; Imhoff and Trüper, 1992). The medium of Nissen and Dundas (1984) is suitable for growth of *Rhodovibrio salinarum* and can also be used for *Rhodovibrio sodomensis*. A recipe for this medium is given below.¹

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen, by lyophilization, or storage at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOVIBRIO* FROM OTHER GENERA

Major differentiating properties for *Rhodovibrio* species and other phototrophic spiral-shaped *Alphaproteobacteria* are shown in Table BXII.α.1 in the description of the genus *Rhodospirillum*.

TAXONOMIC COMMENTS

Two species, *Rhodovibrio salinarum* and *Rhodovibrio sodomensis*, are currently recognized (Imhoff et al., 1998). Because of their spiral shape and phototrophic capacity, *Rhodovibrio* species were originally assigned to the genus *Rhodospirillum* (Nissen and Dundas, 1984; Mack et al., 1993). With growing recognition of the high phenotypic and chemotaxonomic diversity of spiral-shaped phototrophic *Alphaproteobacteria* and the great genetic distance between most of these bacteria, a rearrangement of the species of this group became necessary. Several distinct differences between *Rhodovibrio* species and *Rhodospirillum rubrum* led to their reclassification in the new genus *Rhodovibrio* (Imhoff et al., 1998). The genus name *Rhodovibrio* had originally been proposed by Molisch (1907) for bacteria (*Rhodovibrio parvus*) later recognized as belonging to *Rhodopseudomonas palustris*. Because this name was not included in the Approved List of Names, it had no standing in nomenclature and could be revived (Imhoff et al., 1998). The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A.

1. The medium contains (g/l): NaCl, 80; $MgCl_2 \cdot 6H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $(NH_4)_2SO_4$, 0.5; KH_2PO_4 , 2.5; sodium acetate, 1; sodium glutamate 1; and trace element solution SLA, 1 ml. The initial pH is adjusted to 7.0 (see also Imhoff, 1988).

The mol% G + C of the DNA is: 66.6.

Type species: Roseospira mediosalina (Kompantseva and Gorlenko 1984) Imhoff, Petri and Söling 1998, 798 (*"Rhodospirillum mediosalinum"* Kompantseva and Gorlenko 1984, 780.)

ENRICHMENT AND ISOLATION PROCEDURES

Roseospira mediosalina was isolated from microbial mats of a warm sulfur spring (43–51°C) with elevated mineral salts concentration (2% total salts) and alkaline pH (pH 8.2) (Kompantseva and Gorlenko, 1984). Media and growth conditions applied for *Rhodospirillum* species are also suitable for *Roseospira* species, if appropriate salt concentrations are supplied. A recipe for a mineral salts medium, which can be used if 5% NaCl is added, is given with the description of the genus *Rhodospirillum*. Standard techniques for isolation of anaerobic bacteria in agar dilution series and on agar plates can be applied for *Roseospira* species (Imhoff, 1988; Imhoff and Trüper, 1992). *Roseospira mediosalina* can also be grown on Pfennig's medium for purple sulfur bacteria (see Genus *Chromatium*) with the addition of 5% NaCl and vitamins (or yeast extract).

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen, by lyophilization, or storage at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *ROSEOSPIRA* FROM OTHER GENERA

Major differentiating properties for *Roseospira mediosalina* and other spiral-shaped phototrophic *Alphaproteobacteria* are shown in Table BXII.α.1 and carbon sources utilized in Table BXII.α.2 in the description of the genus *Rhodospirillum*. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are

shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

TAXONOMIC COMMENTS

Because of their spiral shape and phototrophic capacity, *Roseospira* species were originally assigned to the genus *Rhodospirillum*, although DNA homology to *R. rubrum* was insignificant (Kompantseva and Gorlenko, 1984). With growing recognition of the high phenotypic and chemotaxonomic diversity of spiral-shaped phototrophic *Alphaproteobacteria* and the great genetic distance between most of these bacteria (Kawasaki et al., 1993b), a rearrangement of the species of this group became necessary (Imhoff et al., 1998). The great differences between *Roseospira* and *Rhodospirillum* led to their reclassification (Imhoff et al., 1998). The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p. 124) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A.

FURTHER READING

Imhoff, J.F., R. Petri and J. Söling. 1998. Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the *α-Proteobacteria*: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. Int. J. Syst. Bacteriol. 48: 793–798.

Kompantseva, E.I. and V.M. Gorlenko. 1984. A new species of moderately halophilic purple bacterium, *Rhodospirillum mediosalinum*. Mikrobiologiya 53: 954–961.

List of species of the genus *Roseospira*

1. ***Roseospira mediosalina*** (Kompantseva and Gorlenko 1984) Imhoff, Petri and Söling 1998, 798^{VP} (*"Rhodospirillum mediosalinum"* Kompantseva and Gorlenko 1984, 780.) *me' di.o.sa.li' na*. L. adj. *medius* medium; L. neut. n. *salinum* saline, salty; M.L. fem. n. *mediosalina* living at a moderate salinity, moderate halophile.

Cells are vibrioid to spiral shaped, 0.8–1.0 × 2.2–6.0 μm; complete turn of a spiral is 4.0–5.2 μm. They are motile by means of polar flagella and multiply by binary fission. Internal photosynthetic membranes are present as vesicles. Color of cell suspension ranges from pinkish to brownish red. Absorption maxima of living cells occur at 379, 474, 504, 534, 591, 803, and 860 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series.

Growth is preferentially photoheterotrophic in the presence of organic substrates. Photolithoautotrophic growth occurs with sulfide. Sulfide is oxidized to S⁰, which is deposited outside the cell and not further oxidized. Sulfide tolerance is high (up to 2 mM). Thiosulfate does not serve

as an electron donor. Chemotrophic growth is possible under microoxic conditions in the dark in the presence of organic substrates. Fatty acids, butyrate, lactate, pyruvate, amino acids (alanine, aspartate, glutamate), intermediates of the tricarboxylic acid cycle (malate, succinate, fumarate), yeast extract, and Casamino acids are used as carbon sources and electron donors. Benzoate, malonate, tartrate, formate, citrate, alcohols, sugars, and sugar alcohols are not used. Ammonium salts and urea can be utilized as nitrogen sources. Capable of assimilatory sulfate reduction. Storage material is poly-β-hydroxybutyrate. Catalase positive. Thiamine, niacin, and *p*-aminobenzoate are required for growth.

Halophilic bacterium with optimum growth at 30–35°C, pH 7.0, and NaCl concentrations of 4–7% (NaCl range: 0.5–15%). No growth in the absence of salts. No growth above 40°C. Habitat: warm sulfur spring Astara (Azerbaijan) with elevated mineral salts concentration of 2%.

The mol% G + C of the DNA is: 66.6 (*T_m*).

Type strain: BN 280.

GenBank accession number (16S rRNA): AJ000989.

Genus X. Roseospirillum Glaeser and Overmann 2001, 793^{VP} (Effective publication: Glaeser and Overmann 1999, 414)

JOHANNES F. IMHOFF

Ro.se.o.spi.ril' lum. L. adj. *roseus* rosy; M.L. neut. n. *spirillum* the spiral, a bacterial genus; M.L. neut. n. *Roseospirillum* the rosy spiral.

Cells are vibrioid to spiral shaped, motile by means of polar flagella and multiply by binary fission. **Gram negative and belong to the Alphaproteobacteria.** Internal photosynthetic membranes are present as lamellar stacks. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids.

Cells grow photolithoautotrophically and photoheterotrophically under anoxic conditions in the light. Chemotrophic growth is possible under microoxic conditions in the dark. Growth factors are required. Marine bacteria that **require NaCl or sea salt for optimum growth.**

Habitat: marine sediments and microbial mats.

The mol% G + C of the DNA is: 71.2.

Type species: ***Roseospirillum parvum*** Glaeser and Overmann 2001, 793 (Effective publication: Glaeser and Overmann 1999, 414.)

ENRICHMENT AND ISOLATION PROCEDURES

Media and growth conditions applied for *Rhodospirillum* species are also suitable for *Roseospirillum* species, if appropriate salt concentrations are supplied. A recipe of a mineral salts medium that

can be used if 1–2% NaCl is added is given with the description of the genus *Rhodospirillum*. Standard techniques for isolation of anaerobic bacteria in agar dilution series and on agar plates can be applied (Imhoff, 1988; Imhoff and Trüper, 1992).

MAINTENANCE PROCEDURES

Cultures can be preserved by standard techniques in liquid nitrogen, by lyophilization or storage at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *ROSEOSPIRILLUM* FROM OTHER GENERA

Major differentiating properties between *Roseospirillum parvum* and other spiral-shaped phototrophic *Alphaproteobacteria* are shown in Table BXII.α.1, carbon sources utilized in Table BXII.α.2. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 1 (p.124) of the introductory chapter “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A.

List of species of the genus *Roseospirillum*

1. ***Roseospirillum parvum*** Glaeser and Overmann 2001, 793^{VP} (Effective publication: Glaeser and Overmann 1999, 414.) *par'vum*. M.L. neut. adj. *parvum* small.

Cells are vibrioid to spiral shaped, $0.4\text{--}0.6 \times 1.8\text{--}2.6 \mu\text{m}$, motile by means of bipolar flagella and multiply by binary fission. Internal photosynthetic membranes are present as lamellae. Color of a cell suspension is pink to pinkish-red. Absorption maxima of living cells occur at 380, 492, 515, 549, 595, 806, and 911 nm. Photosynthetic pigments are bacteriochlorophyll *a* and the carotenoids spirilloxanthin and lycopenal.

Photolithoautotrophic growth occurs with sulfide and thiosulfate. Sulfide is oxidized to elemental sulfur, which is deposited outside the cells and not further oxidized to sul-

fate. In the presence of sulfide and bicarbonate, acetate, propionate, butyrate, valerate, oxoglutarate, pyruvate, lactate, succinate, fumarate, malate, fructose, Casamino acids, yeast extract, L-alanine, and L-glutamate were assimilated. Chemotrophic growth is possible under microoxic conditions in the dark (with thiosulfate or with thiosulfate and acetate). Incapable of assimilatory sulfate reduction. Yeast extract is required to supply growth factors.

Marine bacterium with optimum growth at 30°C, pH 7.9, and concentrations of 1–2% NaCl (range: up to more than 6% NaCl).

Habitat: microbial mats of Great Sippewissett Salt Marsh. *The mol% G + C of the DNA is:* 71.2 (HPLC).

Type strain: 930I, DSM 12498.

GenBank accession number (16S rRNA): AJ011919.

Genus XI. Skermanella Sly and Stackebrandt 1999, 543^{VP}

THE EDITORIAL BOARD

Sker.ma.nel' la. M.L. dim. *-ella* ending; M.L. fem. dim. n. *Skermanella* named after V.B.D. Skerman, who first isolated this bacterium, and in honor of his contribution to bacterial systematics.

Gram negative, with unicellular and multicellular growth phases. Does not form spores. Unicellular phase cells are rod shaped, with rounded or tapered ends and a straight or curved axis. Motile with single polar flagellum and one or more lateral flagella of different wavelengths. **Multicellular phase conglomerates** arise from cells that lose motility, become optically refractile, and **reproduce by multiplanar septation**. No filamentous structures or buds are formed. Obligately chemoorganotrophic and fac-

ultatively anaerobic, having fermentative metabolism. Belongs to the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 67.2 ± 0.8 .

Type species: ***Skermanella parooensis*** (Skerman, Sly and Williamson 1983) Sly and Stackebrandt 1999, 543 (*Conglomeromonas largomobilis* subsp. *parooensis* Skerman, Sly and Williamson 1983, 307.)

List of species of the genus *Skermanella*

1. ***Skermanella parooensis*** (Skerman, Sly and Williamson 1983) Sly and Stackebrandt 1999, 543^{VP} (*Conglomeromonas largomobilis* subsp. *parooensis* Skerman, Sly and Williamson 1983, 307.)

pa.roo.en'sis. M.L. fem. (and masc.) adj. *parooensis* belonging/pertaining to the Paroo, referring to the Paroo Channel in southwest Queensland, Australia, the source of the water from which the organism was isolated.

Characteristics are as described for the genus, with the following additions. Cells are $1.0\text{--}1.5 \times 1.5 \mu\text{m}$ (peptone yeast extract agar) or $1.0\text{--}1.5 \times 2\text{--}3 \mu\text{m}$ (lake water agar). Noncapsulated. Separated colonies on peptone yeast extract agar are in conglomerate form. Colonies are apricot-colored, opaque, round, and raised, with a rough surface and irregular edge after 72 h at 28°C. Conglomerates have dry appearance and are difficult to emulsify. Confluent growth of single cells is mucoid and emulsifies easily. Conglomerates have the same or greater diameter as colonies of the unicellular form. Colonies grown in the presence of Congo red do not turn scarlet. Aging cells contain poly- β -hydroxybutyrate inclusions. Optimal temperature: 28°C; range 10–37°C. Growth occurs on peptone yeast-extract agar, 0.1% peptone agar, glucose-ammonium sulfate agar,

citrate-ammonium agar, and lake water agar. Slower growth occurs in the presence of 2% NaCl in the conglomerate form. No growth in the presence of 5% NaCl. Catalase, oxidase, aminopeptidase, phosphatase, deoxyribonuclease, and urease are produced. Nitrate reduced to nitrite. Utilizes citrate and malonate. Hydrolyzes Tween 80, but not esculin, chitin, alginate, cellulose, gelatin, or casein. Acid but not gas from cellobiose, galactose, glucose, inositol, lactose, mannitol, mannose, melibiose, and rhamnose. No acid from adonitol, amygdalin, D-arabinose, arabinol, dextrin, dulcitol, erythritol, ethanol, fructose, glycerol, inulin, maltose, melizitose, raffinose, D-ribose, salicin, sorbitol, L-sorbose, starch, sucrose, trehalose, or xylose. Utilizes the following carbon sources: D-arabinose, L-arabinose, D-fructose, gentiobiose, D-galactose, D-glucose, D-lyxose, D-mannitol, D-mannose, melibiose, L-rhamnose, D-ribose, and D-xylose, but not N-acetylglucosamine, D-cellobiose, D-gluconate, glycerol, inositol, lactose, maltose, D-sorbitol, or D-trehalose. The type strain was isolated from fresh water.

The mol% G + C of the DNA is: 67.2 ± 0.8 .

Type strain: ACM 2042, CIP 106994, DSM 9527, UQM 2042.

GenBank accession number (16S rRNA): X90760.

Genus *Incertae Sedis* XII. “*Sporospirillum*” Delaporte 1964, 257

BRUNO POT AND MONIQUE GILLIS

Spor.o.spi.ril' lum. Gr. n. *sporos* a seed (spore); Gr. n. *spira* a spiral; M.L. dim. n. *spirillum* a small spiral; M.L. neut. n. *Sporospirillum* a small spore (-forming) spiral.

Rigid, helical bacteria of enormous size, 1.8–4.8 μm in diameter and 40–100 μm in length. Structures that morphologically resemble endospores occur within the cells, but their thermal resistance has not been determined. **The spore-like structures have the ability to rotate and to migrate within the cytoplasm of the bacteria.** They initially develop near the cell poles and subsequently migrate to the center where they are released after the cell ruptures and disintegrates. The Gram reaction has not been reported. The cells are **motile, but no organs of locomotion are**

evident. The relationship of the cells to oxygen is unknown. Occur in the intestinal contents of tadpoles. Have not been isolated. A type strain has not been designated.

The mol% G + C of the DNA is: not determined.

Type species: “*Sporospirillum bisporum*” Delaporte 1964, 260.

ACKNOWLEDGMENTS

We sincerely acknowledge Dr. N.R. Krieg for kindly providing the template text upon which this chapter was based.

List of species of the genus “*Sporospirillum*”

1. “***Sporospirillum bisporum***” Delaporte 1964, 260.
bi.spo'rum. L. adv. *bis* twice; G. n. *sporos* a seed; M.L. gen. pl. n. *bisporum* of two seeds (spores).

Cell diameter is 3.5–4.8 μm . Cell length is 50–90 μm . Diameter of helix is 11–15 μm . Wavelength is 27–35 μm . An endospore ($2\text{--}4 \times 10\text{--}14 \mu\text{m}$) occurs at each pole.

The mol% G + C of the DNA is: not determined.

Deposited strain: none designated.

2. “***Sporospirillum gyrini***” Delaporte 1964, 259.
gy.ni'ni. L. n. *gyrinus* a tadpole; L. gen. n. *gyrini* of a tadpole.

Cell diameter is 1.8–2.6 μm . Cell length is 40–100 μm . Diameter of helix is 3–6 μm . Wavelength is 13–20 μm . A single endospore is present, $2 \times 5\text{--}7 \mu\text{m}$.

The mol% G + C of the DNA is: not determined.

Deposited strain: none designated.

3. “***Sporospirillum praeclarum***” (Collin 1913) Delaporte 1964, 259 (*Spirillum praeclarum* Collin 1913, 62.)
prae.cla'rum. L. adj. *praeclarum* distinguished, famous.

Cell diameter is 3.0–4.0 μm . Cell length is 50–100 μm . Diameter of helix is 5–10 μm . Wavelength is 17–23 μm . A single endospore is present, $3\text{--}4 \times 9\text{--}12 \mu\text{m}$. A type strain has not been designated.

The mol% G + C of the DNA is: not determined.

Deposited strain: none designated.

Family II. **Acetobacteraceae** Gillis and De Ley 1980, 23^{VP}

MARTIN SIEVERS AND JEAN SWINGS

A.ce.to.bac.te.ra'ce.ae. M.L. masc. n. *Acetobacter* type genus of the family; *-aceae* ending to denote a family; M.L. fem. pl. n. *Acetobacteraceae* the *Acetobacter* family.

The family *Acetobacteraceae*, the Gram-negative, aerobic, acetic acid bacteria, has undergone many taxonomic changes in respect to their genus status. Several species of acetic acid bacteria were newly described. Relationships among the acetic acid bacteria were studied genotypically by DNA–rRNA hybridization, DNA–DNA hybridization, and ribosomal RNA gene sequences (5S rRNA, 16S rRNA, and 23S rRNA). Changes at the generic level in the classification of some acetic acid bacteria are given in Table BXII.α.9. Phenotypic characteristics of six genera are shown in Table BXII.α.10. *Gluconacetobacter*, *Acidomonas*, and *Asaia* as well as *Gluconobacter* species, possess Q-10 ubiquinone as the major quinone component. The Q-10 equipped species contain in addition minor amounts of ubiquinone of the Q-9 type. The ubiquinone system of *Acetobacter pasteurianus*, *Acetobacter pomorum*, and *Acetobacter aceti* is Q-9 along with minor components of ubiquinone of the Q-8 type.

Type genus: Acetobacter Beijerinck 1898, 215.

FURTHER DESCRIPTIVE INFORMATION

Sugar metabolism Acetic acid bacteria catabolize sugars by means of the cytoplasmic hexose monophosphate pathway. Glycolysis is absent due to lack of phosphofructokinase (Leisinger, 1965; Attwood et al., 1991). The Entner–Doudoroff pathway occurs only in cellulose-synthesizing *Gluconacetobacter* strains, where

it appears to be more active than the hexose monophosphate cycle (White and Wang, 1964a, b). The ability to grow on glycerol is due to gluconeogenesis. Specific activities of the key enzymes of the gluconeogenic pathway such as fructose biphosphatase, fructose biphosphate aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase have been described for *Gluconacetobacter diazotrophicus* (Alvarez and Martínez-Drets, 1995). Strains that are able to grow on Hoyer's medium¹ with ethanol as the sole source of carbon and (NH₄)₂SO₄ as the only source of nitrogen utilize the glyoxylate bypass (Leisinger, 1965; De Ley et al., 1984).

Alcohol and aldehyde dehydrogenases of acetic acid bacteria

The oxidizing systems of acetic acid bacteria are shown in Table BXII.α.11. Acetic acid bacteria oxidize ethanol to acetic acid by two successive catalytic reactions of a membrane-bound alcohol dehydrogenase (ADH) and a membrane-bound aldehyde dehydrogenase (ALDH). The enzymes are bound to the cytoplasmic membrane and face the periplasmic space. The ADH and ALDH complexes are tightly linked to the respiratory chain,

1. Hoyer's medium with ethanol consists of (per liter of distilled water): ethanol (99.8 %), 20 ml; (NH₄)₂SO₄, 1.0 g; K₂HPO₄, 0.1 g; KH₂PO₄, 0.9 g; MgSO₄·7H₂O, 0.25 g; and FeCl₃, 0.005 g.

TABLE BXII.α.9. Former and current classification of acetic acid bacteria^a

Former classification	Reference	Current classification	Reference
<i>Acetobacter europaeus</i>	Sievers et al., 1992	<i>Gluconacetobacter europaeus</i>	Yamada et al., 1998b
<i>Acetobacter intermedius</i>	Boesch et al., 1998	<i>Gluconacetobacter intermedius</i>	Yamada, 2000
<i>Acetobacter oboediens</i>	Sokollek et al., 1998b	<i>Gluconacetobacter oboediens</i>	Yamada, 2000
<i>Acetobacter xylinus</i>	Yamada, 1984	<i>Gluconacetobacter xylinus</i>	Yamada et al., 1998b
<i>Acetobacter hansenii</i>	De Ley et al., 1984	<i>Gluconacetobacter hansenii</i>	Yamada et al., 1998b
		<i>Gluconacetobacter entanii</i>	Schüller et al., 2000
<i>Acetobacter diazotrophicus</i>	Gillis et al., 1989	<i>Gluconacetobacter diazotrophicus</i>	Yamada et al., 1998b
		<i>Gluconacetobacter johannae</i>	Fuentes-Ramírez et al., 2001
		<i>Gluconacetobacter azotocaptans</i>	Fuentes-Ramírez et al., 2001
		<i>Gluconacetobacter sacchari</i>	Franke et al., 1999
<i>Acetobacter liquefaciens</i>	Gosselé et al., 1983b	<i>Gluconacetobacter liquefaciens</i> (type species)	Yamada et al., 1998b
<i>Acetobacter aceti</i> (type species)	Beijerinck, 1898	<i>Acetobacter aceti</i> (type species)	Beijerinck, 1898
<i>Acetobacter pasteurianus</i>	Beijerinck, 1916	<i>Acetobacter pasteurianus</i>	Beijerinck, 1916
<i>Acetobacter pomorum</i>	Sokollek et al., 1998b	<i>Acetobacter pomorum</i>	Sokollek et al., 1998b
<i>Acetobacter peroxydans</i>	Visser't Hooft, 1925	<i>Acetobacter peroxydans</i>	Lisdiyanti et al., 2000
<i>Acetobacter estunensis</i>	Carr, 1958	<i>Acetobacter estunensis</i>	Lisdiyanti et al., 2000
<i>Acetobacter lovaniensis</i>	Fratureur, 1950	<i>Acetobacter lovaniensis</i>	Lisdiyanti et al., 2000
<i>Acetobacter orleanensis</i>	Henneberg, 1906	<i>Acetobacter orleanensis</i>	Lisdiyanti et al., 2000
		<i>Acetobacter indonesiensis</i>	Lisdiyanti et al., 2000
		<i>Acetobacter tropicalis</i>	Lisdiyanti et al., 2000
		<i>Acetobacter orientalis</i>	Lisdiyanti et al., 2001a
		<i>Acetobacter cibinongensis</i>	Lisdiyanti et al., 2001a
		<i>Acetobacter syzygii</i>	Lisdiyanti et al., 2001a
<i>Acetobacter methanolicus</i>	Uhlig et al., 1986	<i>Acidomonas methanolica</i> (type species)	Urakami et al., 1989a
		<i>Asaia bogorensis</i> (type species)	Yamada et al., 2000
		<i>Asaia siamensis</i>	Katsura et al., 2001
<i>Gluconobacter oxydans</i> (type species)	De Ley, 1961	<i>Gluconobacter oxydans</i> (type species)	De Ley, 1961
<i>Gluconobacter frateurii</i>	Mason and Claus, 1989	<i>Gluconobacter frateurii</i>	Mason and Claus, 1989
<i>Gluconobacter cerinus</i>	Yamada and Akita, 1984a	<i>Gluconobacter cerinus</i>	Yamada and Akita, 1984a
<i>Gluconobacter asaii</i>	Mason and Claus, 1989	<i>Gluconobacter asaii</i>	Mason and Claus, 1989

^a*Kozakia baliensis* gen. nov., sp. nov. has been published by Lisdiyanti et al., 2002, *International Journal of Systematic and Evolutionary Microbiology* 52: 813–818. *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. have been described by Cleenwerck et al., 2002, *International Journal of Systematic and Evolutionary Microbiology* 52: 1551–1558.

TABLE BXII.α.10. Differential characteristics of the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*^a

Characteristic	<i>Acetobacter</i>	<i>Acidomonas</i>	<i>Asaia</i>	<i>Gluconacetobacter</i>	<i>Gluconobacter</i>	<i>Kozakia</i>
Flagellation	Peritrichous or nonmotile	Nonmotile	Peritrichous or nonmotile	Peritrichous or nonmotile	Polar or nonmotile	Nonmotile
Oxidation of ethanol to acetic acid	+	+	– or w ^b	+	+	+
Oxidation of acetic acid to CO ₂ and H ₂ O	+	+	+	+ ^c	–	w
Oxidation of lactate to CO ₂ and H ₂ O	+	w	+	+ or –	–	w
Growth on 0.35% acetic-acid-containing medium	+	+	–	+	+	+
Growth on methanol	– or w ^d	+	–	–	–	–
Growth on D-mannitol	+ or –	w	+ or –	+ or –	+	+
Growth in the presence of 30% D-glucose	–	–	+	+ or –	– or w	–
Production of cellulose	–	–	–	+ or –	–	–
Production of levan-like mucous substance from sucrose	– or +	–	–	– or +	–	+
Fixation of molecular nitrogen	–	–	–	– or +	–	–
Ketogenesis (dihydroxyacetone) from glycerol	+ or –	w	– or w	+ or –	+	+
Acid production from:						
D-Mannitol	– or +	–	+ or –	+ or –	+	–
Glycerol	– or +	–	+	+	+	+
Raffinose	–	nd	–	–	–	+
Cellular fatty acid type	C _{18:1} ω ₇	C _{18:1} ω ₇	C _{18:1} ω ₇	C _{18:1} ω ₇	C _{18:1} ω ₇	C _{18:1} ω ₇
Ubiquinone type	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10
DNA base composition (mol% G + C)	52–60	63–66	59–61	55–66	54–63	56–57

^aSymbols: +, 90% or more of the strains positive; w, weakly positive reaction; –, 90% or more of the strains negative.^b*Asaia* does not produce acetic acid from ethanol with the exception of one strain producing acid weakly (Yamada et al., 2000).^cOveroxidation of acetate to CO₂ and H₂O depends on acetate concentration in the medium.^d*A. pomorum* assimilates methanol weakly (Sokollek et al., 1998b).**TABLE BXII.α.11.** Oxidizing capacity of acetic acid bacteria

Substrate	Product	Enzyme
Ethanol	Acetic acid	Alcohol dehydrogenase, aldehyde dehydrogenase
<i>iso</i> -Butanol	<i>iso</i> -Butyric acid	Alcohol dehydrogenase, aldehyde dehydrogenase
α-Acetolactate	Acetoin	α-Acetolactate decarboxylase
D-Glycerol	Dihydroxyacetone	Glycerol dehydrogenase
D-Mannitol	D-Fructose	D-Mannitol dehydrogenase
D-Sorbitol	L-Sorbose	D-Sorbitol dehydrogenase
D-Glucose	D-Gluconic acid	D-Glucose dehydrogenase
D-Gluconic acid	2-Keto-D-gluconic acid	D-Gluconate dehydrogenase
D-Gluconic acid	5-Keto-D-gluconic acid	D-Gluconate dehydrogenase
2-Keto-D-gluconic acid	2,5-Diketo-D-gluconic acid	2-Keto-D-gluconate dehydrogenase
L-Sorbose	L-Sorbose	L-Sorbose dehydrogenase
L-Sorbose	2-Keto-D-gulononic acid	L-Sorbose dehydrogenase
D-Fructose	5-Keto-D-fructose	D-Fructose dehydrogenase

which transfers electrons via ubiquinone and a terminal ubiquinol oxidase to oxygen as final electron acceptor.

The ADH complex in acetic acid bacteria is composed of two or three subunits. The molecular weight of the subunits estimated by SDS-PAGE are 72, 44, and 15 kDa in *Gluconobacter oxydans* (Kondo and Horinouchi, 1997b), 72, 50, and 15 kDa in *Acetobacter aceti* (Inoue et al., 1989, 1992; Matsushita et al., 1992d), 78, 48, and 20 in *Acetobacter pasteurianus* (Kondo et al., 1995), 80, 54, and 8 kDa in *Acidomonas methanolic* (Frébortová et al., 1997), 72 and 44 kDa in "*Acetobacter polyoxogenes*" (Tayama et al., 1989; Tamaki et al., 1991), and 80 and 49 kDa in *Gluconacetobacter europaeus* (Thurner, 1997). The larger subunit of the ADH possesses a heme C and a pyrroloquinoline quinone (PQQ) as cofactors and requires Ca²⁺ to be active, according to the catalytic mechanism given by Anthony (1996) and Goodwin and Anthony (1998). The second subunit is a cytochrome *c*. The third and smallest subunit of the three-component ADH is discussed to protect the catalytic subunit from proteolysis and to keep the correct conformation of the ADH complex for electron transport on the periplasmic surface (Kondo et al., 1995). The genes encoding the dehydrogenase subunit and the cytochrome *c* sub-

unit of ADH are clustered in the same transcription polarity and were cotranscribed. The cytochrome *c* subunit of the ADH complex of *Gluconobacter oxydans* has an additional function: it is a component of the non-energy-generating cyanide-insensitive bypass terminal oxidase in the respiratory chain (Matsushita et al., 1994). Thus, the cytochrome *c* subunit gene of *Gluconobacter oxydans* is transcribed independently from the dehydrogenase subunit gene (Matsushita et al., 1989). The ADH activity of *Acetobacter pasteurianus* is induced by ethanol. The ADH gene cluster utilizes two different promoters for its expression, in response to the presence or absence of ethanol in the culture medium (Take-mura et al., 1993). Since ethanol is the major substrate for energy generation in acetic acid bacteria through the ADH and ALDH reactions, the role of the function of ethanol in regulatory mechanisms is to be elucidated. Transcription of the gene encoding the enzyme arylesterase, which is related in ethylacetate formation from acetic acid and ethanol, is induced by ethanol in *Acetobacter pasteurianus* (Kashima et al., 1999). In vinegar, esters weaken the strong smell of acetic acid (Kashima et al., 1999).

The ALDH complex of acetic acid bacteria, which catalyzes the oxidation of acetaldehyde to acetic acid, is composed of two

or three subunits. The molecular masses are 86 and 55 kDa for *Gluconobacter oxydans*, 78, 45, and 14 kDa for *Acetobacter aceti*, and 75 and 19 kDa for "*A. polyoxogenes*" (Tamaki et al., 1989; Matsushita et al., 1994). The ALDH from *Gluconacetobacter europaeus* is composed of three subunits with molecular masses of 79, 49, and 17 kDa. The aldehyde dehydrogenase complex is organized as an operon. The larger catalytic subunit contains heme *b* and a pterin molybdenum instead of PQQ. The middle subunit is a cytochrome *c* with three heme *c* binding sites. The smallest subunit contains two [2Fe-2S] clusters (Thurner, 1997; Thurner et al., 1997). The catalytic subunit of the ALDH of *Gluconacetobacter europaeus* differs by nine amino acids from the corresponding protein of "*A. polyoxogenes*". Each gene of the ADH and ALDH subunits contains a signal sequence encoding for a leader peptide, responsible for translocation of the proteins through the cytoplasmic membrane. The ADH and ALDH of acetic acid bacteria have a broad substrate specificity and oxidize straight and branched chain alcohols and aldehydes into the corresponding carboxylic acids.

Alcohol and aldehyde dehydrogenases dependent on NAD(P)⁺ have been isolated and characterized from the cytoplasm of acetic acid bacteria. The NAD(P)⁺-dependent enzymes show an activity optimum at alkaline pH with much lower specific activities than those of quinoprotein enzymes, which show activity optimum at acidic pH (Matsushita et al., 1994).

Aldehydes produced during food processing can react with amino groups to form colored materials. ADH can scavenge aldehydes and carbonyl residues and since carboxylic acids react little with amino acids, the addition of acetic acid bacteria prevents coloration of food and reduces off-flavors (Nomura et al., 1995a). For instance, to reduce the stale flavor of cooked rice, rice grains have been incubated with a freeze-dried powder of acetic acid bacteria at 50°C, which resulted in a decrease of *n*-hexanol in the rice (Nomura et al., 1995b). Another example of the use of acetic acid bacteria to reduce off-flavors in food involves the cloning and expression of the gene that encodes α -acetolactate decarboxylase from *Gluconacetobacter xylinus* in brewer's yeast: during beer fermentation, brewer's yeasts produce α -acetolactate, which is converted to diacetyl by slow non-enzymatic oxidative decarboxylation. Diacetyl causes off-flavors in beer. The α -acetolactate decarboxylase of *Gluconacetobacter xylinus* converts the α -acetolactate directly to acetoin, which has no effect on the flavor of beer (Yamano et al., 1994).

Acetobacter, *Gluconacetobacter*, and *Acidomonas* are, in contrast to *Gluconobacter*, equipped with a complete tricarboxylic acid (TCA) cycle. The amount of TCA cycle enzymes in cells is correlated with the energy requirement of the cells (Rault-Leonardon et al., 1995). Key enzymes of the TCA cycle are influenced by different intermediate metabolites; an example is the citrate synthase of *Gluconacetobacter europaeus*, which is activated by acetate (Sievers et al., 1997). *Gluconacetobacter* and *Acetobacter* species producing high concentrations of acetic acid are not able to keep the internal pH constant and need additional ATP for internal protection against acetate and H⁺ (Menzel and Gottschalk, 1985). Bacteria have to use acid-resistant systems like genes that are induced by environmental stimuli even if the cytoplasm is acidified by low external pH (Kobayashi et al., 2000). As shown for *Acetobacter aceti* and *Gluconobacter oxydans*, eight acetate-specific stress proteins (Asps) were overexpressed in both species during growth in the presence of acetate. Three Asps were similar and five Asps were different in both species analyzed by two-dimensional gel electrophoresis of total protein extracts (Lasko et al.,

1997). The acetate-activating enzyme, acetyl-CoA synthetase, has been purified from the cytoplasm of *Gluconacetobacter europaeus* and *Gluconobacter oxydans*. The native acetyl-CoA synthetase of *Gluconacetobacter europaeus* and *Gluconobacter oxydans* has a molecular mass near 150 kDa and is composed of two identical subunits with a molecular mass near 75 kDa, suggesting that the enzyme is a dimer. Due to the instability of the enzyme, 15% glycerol was included in the buffer system used. The activity of the acetyl-CoA synthetase was inhibited by AMP, ADP, and Na-citrate (M. Sievers, F. Tschudin, and M. Teuber, unpublished results). The activity of this enzyme is increased significantly in *Acetobacter* cells when acetate is consumed. Addition of glycerol to the culture medium of "*Acetobacter rancens*" increased acetate oxidation (Saeki et al., 1999). The genes responsible for acetic acid resistance in *Acetobacter aceti* are described by Fukaya et al. (1990) and Fukaya et al. (1993).

Respiratory chains Acetic acid bacteria are oxidase negative, with the exception of *Acidomonas methanolica*, which is oxidase positive during growth on methanol. The respiratory chain in *Gluconobacter* consists of cytochrome *c*, ubiquinone, and cytochrome *o* as a terminal ubiquinol oxidase; it also has a cyanide-insensitive alternative terminal ubiquinol oxidase. The respiratory chain in *Acetobacter* and *Gluconacetobacter* has cytochrome *c*, ubiquinone, and a terminal ubiquinol oxidase of the cytochrome *a₁*, cytochrome *d*, or cytochrome *o* type (Matsushita et al., 1994). The respiratory chain of *Acidomonas methanolica* contains two different terminal oxidases, viz. a cytochrome *c* oxidase and an ubiquinol oxidase (Matsushita et al., 1992c). Under methylo-trophic conditions, *Acidomonas methanolica* produces a cytochrome *co* terminal cytochrome *c* oxidase (Chan and Anthony, 1991). When grown under nonmethylo-trophic conditions, however, *Acidomonas methanolica* produces cytochrome *o* as the terminal ubiquinol oxidase (Matsushita et al., 1994). Cytochrome *o* from *Acetobacter aceti* and *Acidomonas methanolica* contains heme *o* (Puustinen and Wikström, 1991) and heme *b* in a 1:1 ratio (Matsushita et al., 1992b). One cell type of *Acetobacter aceti* forms smooth-surfaced colonies, while the other cell type of *Acetobacter aceti* forms rough-surfaced colonies. Cells of the former type predominate in shaking cultures, while cells of the latter type predominate in static cultures. There is a difference in the terminal oxidase, which is cytochrome *a₁* in cells in shaking culture, but cytochrome *o* in cells grown statically (Matsushita et al., 1992a, 1994). Cytochrome *a₁* has a higher affinity for oxygen than cytochrome *o* (Matsushita et al., 1992b), which results in enhanced growth of smooth-surfaced cells of *Acetobacter aceti* in shaking culture. The two different types of colonies of *Acetobacter aceti* with their corresponding types of cytochrome are shown in Fig. BXII.α.15 (Matsushita et al., 1994).

α -Amino acid ester hydrolase The α -amino acid ester hydrolase from "*Acetobacter turbidans*" ATCC 9325 is a beta-lactam antibiotic acylase capable of hydrolyzing beta-lactam antibiotics (cephalexin, ampicillin). The enzyme also catalyzes the transfer of the acyl group from α -amino acid esters to 7-aminocephem and 6-penam compounds. "*Acetobacter turbidans*" ATCC 9325 was described by Takahashi et al. (1972, 1974) as an acetic acid bacterium able to synthesize cephalosporins. The gene *aehA* (AF439262) encodes a dimeric protein with subunits of 70 kDa and an N-terminal leader sequence of 40 amino acids. The α -amino acid ester hydrolase has the motif GXSYXG in the active site, which is found in serine proteases, suggesting that the enzyme is a serine hydrolase (Polderman-Tijmes et al., 2002).

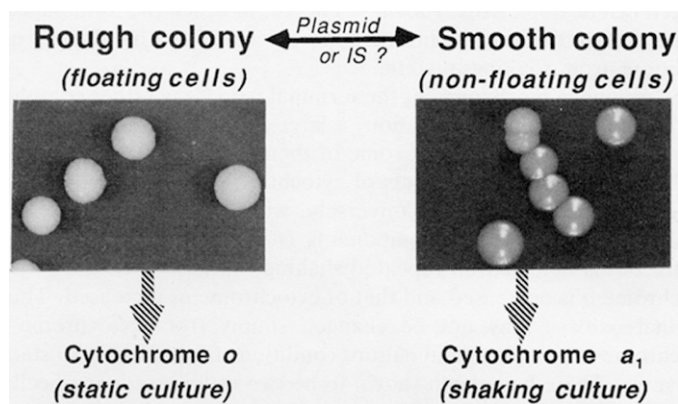


FIGURE BXII.15. Two different types of colonies of *Acetobacter aceti* from cells grown in static and shaking cultures. Cells forming rough-surfaced colonies can grow floating on the surface of the culture and thus predominate in static cultures. Cells forming smooth-surfaced colonies are incapable of floating and thus grow only in shaking cultures. The two types of cells produce cytochrome *o* and cytochrome *a*₁, respectively, as the terminal oxidase. (Reproduced with permission from K. Matsushita et al., *Advances in Microbial Physiology*, 36: 247–301, 1994, ©Academic Press Limited, London.)

Production of cellulose and acetan The production of cellulose by *Gluconacetobacter xylinus* has been regarded as the model system for the study of biochemistry and genetics of cellulose biogenesis and is described by Ross et al. (1991), Cannon and Anderson (1991), and Delmer and Amor (1995). The rate of cellulose production in *Gluconacetobacter xylinus* is proportional to the rate of cell growth, and the yield is dependent on the source of carbon (Embuscado et al., 1994; Oikawa et al., 1995). The cellulose production by *Gluconacetobacter xylinus* strains is enhanced by the addition of small amounts of cellulase (endo- β -1,4-glucanase) from *Bacillus subtilis* to the production culture and the amount of produced acetan is reduced (Tonouchi et al., 1995). Activators for the bacterial cellulose production were compounds like caffeine and related xanthines. The suggested target for these activators was the diguanyl cyclic phosphodiesterase whose inhibition favors the cellulose biosynthesis (Fontana et al., 1991). The produced cellulose is of high purity. Products from bacterial cellulose include wound dressings, skin substitutions, high-quality additives for paper, fiber glass filter sheets, chewing gum, food stabilizers, and acoustic diaphragms for audio instruments (Ross et al., 1991). A mechanical separation method and an alkali treatment for the isolation of bacterial cellulose with removal of bacterial cells were developed (Embuscado et al., 1996).

Gluconacetobacter xylinus secretes long β -1,4 glucan chains in a diameter of 1.5 nm from pores along the longitudinal axis of the cell. These glucans aggregate into 3–4-nm microfibrils by crystallization with subsequent assembly to ribbons in the surrounding medium (Cannon and Anderson, 1991; Ross et al., 1991). The membrane-bound cellulose synthase uses UDP-glucose directly as a substrate and catalyzes the polymerization of the glucose residues to β -1,4 glucan (Saxena et al., 1990). The genes coding for cellulose production are organized in the form of an operon. An operon (*acs*) consisting of three genes is functional for the final steps in cellulose biosynthesis in *Gluconacetobacter xylinus* ATCC 53582. The first gene (*acsAB*) codes for the 168-kDa cellulose synthase, the second gene (*acsC*) codes for a

138-kDa pore-protein, and the third gene (*acsD*) of the operon codes for a 17-kDa protein, which is involved in the crystallization of the microfibrils (Saxena et al., 1994). An additional gene encoding cellulolytic activity (*acsAII*) that is similar to the *acsAB* gene product was detected upstream of the cellulose-synthesizing operon in a *Gluconacetobacter xylinus* strain (Saxena and Brown, 1995). In the *bcs* operon from *Gluconacetobacter xylinus* 1306-3 and BPR 2001 a difference in the numbers of open reading frames coding for cellulose synthase is observed in comparison to the *acs* operon (Wong et al., 1990; Nakai et al., 1998). The first two of the four open reading frames of the *bcs* operon encodes the cellulose synthase subunit A (*bcsA*) and B (*bcsB*). In addition, two open reading frames ORF1 and ORF2 are located upstream of the *bcs* operon. The gene product of ORF1 is identified as cellulase (Standal et al., 1994). The presence of cellulase enhances the production of cellulose, and the integration of IS 1031A in ORF2 causes the inability to produce cellulose. The promoter of the *bcs* operon upstream from *bcsA* and downstream from ORF1 encoding CMCase is overlapping with the ORF2 (Nakai et al., 1998).

The cellulose synthase is activated by cyclic diguanylic acid (c-di-GMP), which acts as an allosteric effector and stimulates the enzyme reaction rate up to 200-fold (Ross et al., 1987). The enzymes diguanylate cyclase and phosphodiesterase A catalyze the synthesis and degradation, respectively, of c-di-GMP and thus have regulatory effects on cellulose biosynthesis. The genes encoding isoenzymes for phosphodiesterase A and diguanylate cyclase are organized on three unlinked homologous operons (Tal et al., 1998).

A conserved UDP-glucose/UDP-N-acetylglucosamine binding motif exists among glycosyltransferases using UDP-glucose or UDP-N-acetylglucosamine as a substrate (Delmer and Amor, 1995). Cotton and rice contain amino acid sequences homologous to bacterial sequences of the catalytic part of the cellulose synthase (Pear et al., 1996).

Gluconacetobacter xylinus synthesizes a cellulose mat, which covers the surface of the growth medium in static cultures, whereas round balls of cellulose are formed in shaking cultures. Aeration of cultures of *Gluconacetobacter xylinus* strains by stirring or shaking gives rise to the formation of spontaneous non-cellulose-producing mutants (Ross et al., 1991). This phenomenon is also observed by *Gluconacetobacter europaeus* strains. The Cel[−] mutants are stable during passages of cultivations in static and shaken cultures.

Acetan, an acidic exopolysaccharide that is related to xanthan, has been isolated from the culture medium of *Gluconacetobacter xylinus* (Couso et al., 1987). Acetan contains glucose, mannose, glucuronic acid, and rhamnose in a molar ratio of 4:1:1:1. The described structure is composed of a pentasaccharide side chain Rha(1-6)Glc (β 1-6)Glc(α 1-4)Glc(β 1-2)Man, which is α -1,3-linked to every second glucose residue of the β -1,4 glucan chain (Couso et al., 1987). Genes encoding UDP-glucose dehydrogenase, UDP-glycosyl transferase, GDP-mannosyl transferase, and phosphomannose isomerase/GDP-mannose pyrophosphorylase of the acetan biosynthetic pathway have been identified and sequenced from *Gluconacetobacter xylinus* (Griffin et al., 1994, 1996, 1997; Petroni and Ielpi, 1996).

Nitrogen fixation Diazotrophy, the ability to fix free nitrogen gas (N₂) into cell material by reducing it to ammonium, occurs in species of more than 100 genera of *Bacteria* and *Archaea*. Proteins for nitrogen fixation (*nif*) have common structures and

functions, while the degree of linkage and arrangement of specific *nif* and associated genes vary considerably in many diazotrophs (Lee et al., 2000). Among the acetic acid bacteria, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae*, and *Gluconacetobacter azotocaptans* were able to fix molecular nitrogen. The *nifH* gene sequence encoding the Fe protein of the nitrogenase and *nifHDK* genes of *Gluconacetobacter diazotrophicus* are highly similar to those of other nitrogen-fixing bacteria, particularly with other diazotrophic members of the *Alphaproteobacteria* (Franke et al., 1998; Sevilla et al., 1998). The *nifA* and *nifB* genes are adjacent and located upstream from the *nifHDK* genes. The *nifA* gene encoding the transcriptional activator for expression of *nif* genes is repressed at high ammonium concentrations (Teixeira et al., 1999). The cluster of *nif*, *fix*, and associated genes of *Gluconacetobacter diazotrophicus* is about 30.5 kb in size and encodes for 33 proteins with a molecular mass sum up to 1095.5 kDa (Lee et al., 2000). Individual gene products of the cluster of *nif*, *fix*, and associated genes like *mcpA*, which is involved in chemotaxis, are similar to those in species of *Rhizobiaceae* or in *Rhodobacter capsulatus* (Lee et al., 2000).

Insertion sequences Insertion sequences integrated in the genome of acetic acid bacteria are responsible for genetic instability leading to deficiencies in physiological properties like inactivation of alcohol dehydrogenase and loss of cellulose and acetan synthesis. IS elements characterized from acetic acid bacteria are listed in Table BXII.α.12.

The reported IS elements are widely distributed in acetic acid bacteria. Their copy numbers are from 1 to about 16 with the exception of IS1380, whose copy number is about 100. The distribution of IS elements in *Gluconacetobacter xylinus*, *Acetobacter pasteurianus*, *Acetobacter aceti*, *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, "*A. polyoxogenes*", *Gluconobacter oxydans*, and *Gluconobacter cerinus* indicates frequent horizontal and vertical gene transfer between strains of acetic acid bacteria. The IS 1031 family, consisting of IS1031A to IS1031D (Coucheron, 1993) and the related IS elements IS 1032 (Iversen et al., 1994) and IS12528

(Kondo and Horinouchi, 1997a) are members of the heterogeneous IS5 group in the higher IS4 family based on conserved transposase motifs (Rezsöházy et al., 1993). Additional insertion elements are present in the chromosome of *Acetobacter pasteurianus* NCIB 7214. The strain contains five copies of IS12528 (Kondo and Horinouchi, 1997a), 10 copies of IS 1452 (Kondo and Horinouchi, 1997c), and 100 copies of IS1380 (Takemura et al., 1991) with a total insertion element length of 188.5 kb, which is over 6% of the nucleotide sequence of the genome (Kondo and Horinouchi, 1997a). Similarly, *Acetobacter aceti* 1023 contains one copy of IS12528, three copies of IS1452, and 100 copies of IS1380 with a total length of 175 kb, corresponding to 6% of the chromosome (Kondo and Horinouchi, 1997a).

Plasmids and phages A variety of plasmids has been reported from strains of *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*, and genetic transformation systems and plasmid vectors for acetic acid bacteria have been established. An overview of the genetics of *Acetobacter* with respect to acetic acid fermentation has been given by Beppu (1993). Conjugal transfer systems by using broad host-range plasmids based on RK2 replicon as vectors for acetic acid bacteria have been described (Valla et al., 1985; Blatny et al., 1997). For example, plasmid p21R1 (RK2-derivate carrying the gene encoding for levansucrase) was introduced in *Gluconacetobacter diazotrophicus*. In the recombinant bacterium, levansucrase production was increased about fourfold due to the low copy number of p21R1 (Hernández et al., 1999). Electroporation systems for *Gluconobacter oxydans* (Creaven et al., 1994) and *Gluconacetobacter xylinus* (Hall et al., 1992) with heterologous plasmids that gave transformation frequencies of up to 10^5 transformants/ μ g of DNA have been developed. An efficient electroporation method for transformation of "*A. polyoxogenes*" with plasmid DNA was adapted to the cultivation parameters of this organism (Tayama et al., 1994).

Introduction of the ALDH gene from "*A. polyoxogenes*" in *Gluconacetobacter xylinus* enhanced the production of acetic acid by overexpression of the cloned gene (Fukaya et al., 1989). The

TABLE BXII.α.12. Insertion sequences of acetic acid bacteria

IS element	Strain	GenBank accession number	Mutant deficiency	Insertion	Target site of duplication ^a	bp	Terminal inverted repeat bp	Reference
IS1380	<i>Acetobacter pasteurianus</i> (NC11380)	D90424	Alcohol oxidation	Cytochrome <i>c</i>	TCGA	1665	15	Takemura et al. (1991)
IS1031A ^b	<i>Gluconacetobacter xylinus</i> ATCC 23769	M80805	Cellulose production	ORF 2, 500 bp upstream of the operon for cellulose biosynthesis	TGA	930	24	Coucheron (1991), Standal et al. (1994)
IS1032	<i>Gluconacetobacter xylinus</i> ATCC 23770	U02294	Acetan production	Gene required for acetan production	TCA	916	IR-Left: 14, IR-Right: 16	Iversen et al. (1994)
IS1452	<i>Acetobacter pasteurianus</i> (NC11452)	D63923	Alcohol oxidation	Subunit III of the alcohol dehydrogenase complex	CTAR	1411	21	Kondo and Horinouchi (1997c)
IS12528	<i>Gluconobacter oxydans</i> IFO 12528	D86632	Alcohol oxidation	Dehydrogenase subunit of the alcohol dehydrogenase complex	TMA	904	18	Kondo and Horinouchi (1997a)

^aPreferred target site M = A,C; R = A,G.

^bIS1031A is 87.1% homolog to IS1031C and IS1031D from *G. xylinus* ATCC 23769 (Coucheron, 1993).

1440-bp plasmid pAP12875 isolated from *Acetobacter pasteurianus* contains two open reading frames, of which one has similarity to the replicon protein of pVT736-1 from *Actinobacillus actinomycetemcomitans* and the 32-kDa protein of phage Pf3 from *Pseudomonas aeruginosa* (Fomenkov et al., 1995). Plasmid pAH4 was characterized from a cellulose-producing *Acetobacter* (*Gluconacetobacter*) strain; it consisted of 4002 bp, contained an A-T rich region, and encoded four open reading frames. A shuttle vector system of *Escherichia coli* and this strain was constructed by connecting pAH4 to pUC18 (Tonouchi et al., 1994). Mobilization regions of plasmid pAEU601 (3818 bp) from *Gluconacetobacter europaeus* DSM 6160 have DNA similarities to plasmids RSF1010 from *E. coli* and pTF1 from *Thiobacillus ferrooxidans*, contributing to the broad distribution of pAEU601 in *Gluconacetobacter europaeus* strains via conjugation events (Boesch, 1998). Shuttle vectors (Amp^r) for cloning and expression of genes in *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, and *E. coli* were constructed by ligation of pJK2-1 from *Gluconacetobacter europaeus* and pUC18 (Trček et al., 2000).

Lysogenic phage Acm1 from *Acidomonas methanolica* has been described and characterized (Wünsche et al., 1983; Mamat et al., 1995). Phage particles from vinegar fermentations, with hexagonal and icosahedral heads, were morphologically characterized and proved to belong to group A and C of Bradley (Bradley, 1967), respectively (Teuber et al., 1987a; Stamm et al., 1989; Defives et al., 1990; Sellmer et al., 1992). Seven morphologically different types of phage particles from vinegar fermentations with hexagonal heads were identified based on head size, tail length, and diameter by Sellmer et al. (1992). Head sizes varied between 60 and 110 nm, the corresponding tail length ranged from 99 to 360 nm, and tail diameter ranged from 14 to 31 nm (Sellmer et al., 1992). *Gluconacetobacter europaeus* does not grow well in soft agar; consequently, the classical plaque test is not suitable to enumerate infective bacteriophages from vinegar fer-

mentations (Sievers and Teuber, 1995). Restriction-modification systems have been detected in *Acetobacter* and *Gluconacetobacter* strains and further characterized (Tagami et al., 1988; Suzuki et al., 1996; Coucheron, 1997; Nwankwo et al., 1997).

TAXONOMIC COMMENTS

Phylogenetic affiliations of *Acetobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, and *Gluconobacter* species and related acidophilic bacteria have been studied on the basis of 5S (Bulygina et al., 1992) and 16S rRNA sequences (Sievers et al., 1994a, 1995a; Kishimoto et al., 1995b; Yamada et al., 1997, 2000; Sokollek et al., 1998b; Franke et al., 1999; Schüller et al., 2000; Lisdiyanti et al., 2001a). All of the 16S rRNA sequences show nucleotide deletions in their loop helices V1, V2, and V3, which are characteristic for the *Alphaproteobacteria*. The clustering of *Gluconacetobacter*, *Acetobacter*, and *Gluconobacter* in the *Alphaproteobacteria* had already been shown by Gillis and De Ley (1980) based on RNA-DNA hybridization studies. All species of the six genera of acetic acid bacteria *Acetobacteraceae*, together with *Acidisphaera rubrifaciens*, *Rhodopila globiformis*, *Acidocella*, *Acidiphilium*, and *Roseococcus thiosulfatophilus* as phylogenetic neighbors, formed a cluster with a distinct line of descent (Fig. BXII.α.16). These organisms were characterized by an acidophilic phenotype with the exception of *Roseococcus thiosulfatophilus*. These acidophilic bacteria became adapted to acidic environments after branching off from the common ancestor of the *Alphaproteobacteria* (Kishimoto et al., 1995b). A phylogenetic tree reflecting the close relationships of *Acetobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, and *Gluconobacter* based on the maximum parsimony analysis is shown in Fig. BXII.α.17. Phylogenetically, the acetic acid bacteria form four major clusters, one containing the *Gluconacetobacter* species with a subcluster comprising *Gluconacetobacter europaeus*, *Gluconacetobacter xylinus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter entanii*, *Gluconacetobacter hansenii*, and one

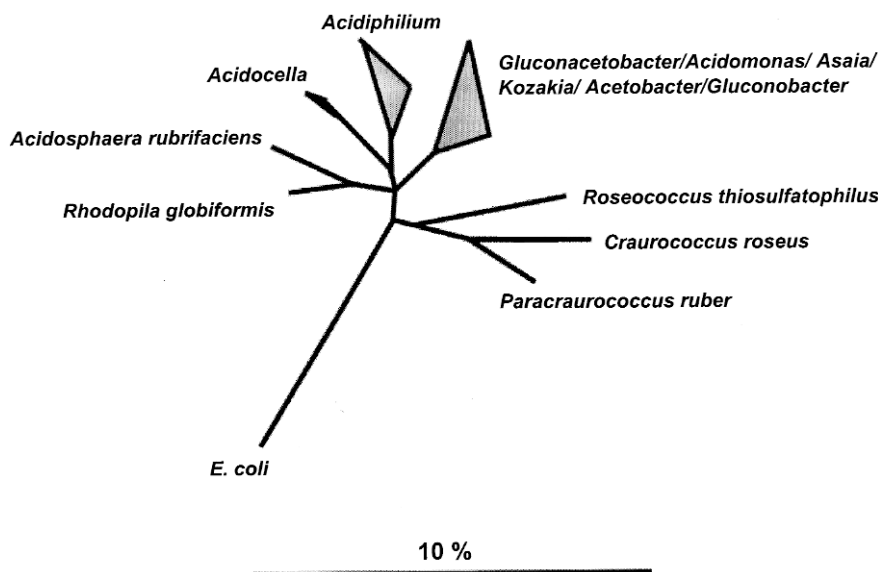


FIGURE BXII.α.16. Phylogenetic tree based on maximum parsimony analysis reflecting the distant relationships of the *Acetobacter*–*Gluconobacter* cluster and their close relatives among members of the *Alphaproteobacteria*. The topology of the tree was evaluated by applying distance matrix and maximum likelihood analyses. The lengths of the edges indicate the extremes of high and low phylogenetic distances among the members of the particular group. The bar indicates 10% estimated sequence difference. (Courtesy of Wolfgang Ludwig, TU Munich, Germany.)

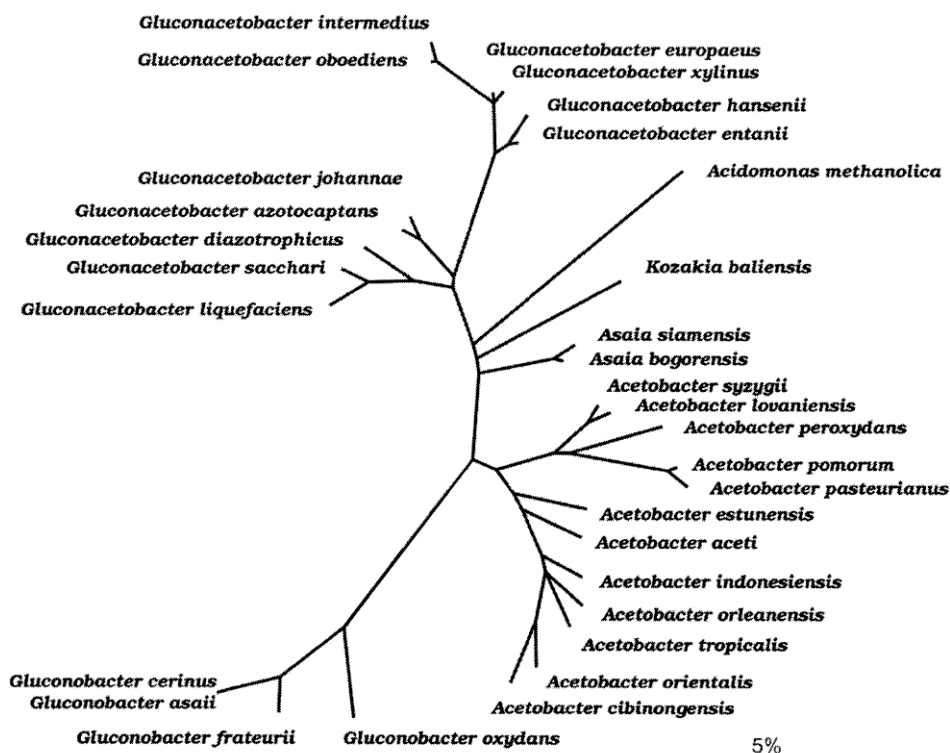


FIGURE BXII.α.17. Phylogenetic tree reflecting the close relationships of the species *Gluconacetobacter*, *Acidomonas methanolica*, *Asaia*, *Acetobacter*, and *Gluconobacter*. The tree is based on the results of a maximum parsimony analysis. The topology of the resulting tree were evaluated by applying the treeing methods (distance matrix, maximum parsimony, and maximum likelihood) to various data sets. The bar indicates 5% estimated sequence difference. (Courtesy of Wolfgang Ludwig, TU Munich, Germany.)

subcluster comprising *Gluconacetobacter sacchari*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae*, and a second cluster containing *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Acetobacter peroxydans*, *Acetobacter pomorum*, *Acetobacter pasteurianus*, *Acetobacter estunensis*, *Acetobacter aceti*, *Acetobacter indonesiensis*, *Acetobacter orleanensis*, *Acetobacter tropicalis*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, and a third cluster comprising *Gluconobacter oxydans*, *Gluconobacter asaii*, *Gluconobacter cerinus*, and *Gluconobacter frateurii*. *Kozakia baliensis* formed a sublineage separated from *Asaia bogorensis* and *Asaia siamensis*. *Acidomonas methanolica* represents a distinct phylogenetic line and the separate branching of this organism is supported by the majority of the treeing analyses. The overall 16S rRNA sequence similarity values of the type species of five genera of acetic acid bacteria are given in Table BXII.α.13. The 16S rRNA sequence similarity between the species of the genus *Gluconacetobacter* is above 96.5%. *Gluconacetobacter europaeus*, *Gluconacetobacter xylinus*, *Gluconacetobacter intermedius*, and *Gluconacetobacter oboediens* are closely related by sharing over 99% 16S rRNA sequence similarities. Within the genus *Acetobacter*, the overall 16S rRNA sequence similarities are above 95.8% and less than 96.6% with those of other genera. Within the genus *Gluconobacter*, the overall 16S rRNA sequence similarities are 97.0–98.8%. *Gluconobacter* is well separated from the other genera by numerical analyses of protein gel electrophoregrams (Gosselé et al., 1983a, b; Sievers and Teuber, 1995) and by phenotypic features (Gosselé et al., 1983a, b). Two cellulose-producing *Gluconacetobacter* strains (ITDI 2.1 and PA 2.2) involved in nata de coco production were characterized by Bernardo et al. (1998).

Based on 16S rDNA sequence analysis, strain ITDI 2.1 is closely related to *Gluconacetobacter xylinus* and strain PA 2.2 to *Gluconacetobacter hansenii*; these strains are discussed as new subspecies under these species designations (Bernardo et al., 1998).

The 16S–23S rDNA intergenic spacer regions of *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, and *Gluconacetobacter xylinus* contain genes encoding for two tRNA molecules specific for L-isoleucine and L-alanine. The tRNA^{Ile} and tRNA^{Ala} sequences are identical in these species. *Gluconacetobacter* seems to contain four copies of the *rrn* operons on its chromosome. Downstream from the tRNA^{Ala} gene a boxA with the nucleotide sequence TGCTCTTTGATA and a putative boxB consisting of 30 nucleotides with short inverted repeats are present in the spacer regions of these species (Sievers et al., 1996). These antitermination sequences are necessary to prevent premature termination of the precursor rRNA (Condon et al., 1995).

PCR-RFLP of the 16S rDNA with *TaqI* and *RsaI* allows identification of acetic acid bacteria on genus level and in some cases at species level (Ruiz et al., 2000). 16S–23S rDNA intergenic spacer restriction patterns can be used to identify strains of acetic acid bacteria due to the variability of these sequences. The type strains of *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, and *Gluconacetobacter xylinus* with a high percentage of 16S rDNA similarity have different sequences of their 16S–23S rDNA intergenic spacer regions (Sievers et al., 1996).

For characterization of cultures producing high percentage spirit, wine, and cider vinegar, plasmid profiling has been used to allow definite conclusions regarding origin, stability, and composition of the *Gluconacetobacter europaeus* strains isolated from

TABLE BXII.α.13. Overall 16S rRNA sequence similarity values for *Gluconacetobacter*, *Asaia*, *Acidomonas*, *Acetobacter*, and *Gluconobacter* species

% 16S rRNA sequence similarity															
Species	<i>Acetobacter aceti</i>	<i>Acetobacter cibinongensis</i>	<i>Acetobacter estuensis</i>	<i>Acetobacter indonesiensis</i>	<i>Acetobacter lovaniensis</i>	<i>Acetobacter orientalis</i>	<i>Acetobacter orleanensis</i>	<i>Acetobacter pasteurianus</i>	<i>Acetobacter peroxydans</i>	<i>Acetobacter pomorum</i>	<i>Acetobacter syzygii</i>	<i>Acetobacter tropicalis</i>	<i>Acidomonas methanohila</i>	<i>Asaia bogorensis</i>	<i>Asaia stamensis</i>
<i>Acetobacter aceti</i>	100	98	97.9	98	97.2	97.8	97.8	96.8	96.6	97.3	97.5	97.9	95.2	96.5	96.3
<i>Acetobacter cibinongensis</i>	98	100	97.7	98.6	97.4	99	98.1	96.8	96.8	97.2	97.6	98.4	94.7	95.7	95.5
<i>Acetobacter estuensis</i>	97.9	97.7	100	97.5	97.1	97.4	97.5	96.3	97.1	96.6	97.3	97.4	94.9	96.6	96.4
<i>Acetobacter indonesiensis</i>	98	98.6	97.5	100	97.1	98.3	98.3	96.4	96.7	96.7	97.3	98.5	94.7	95.7	95.7
<i>Acetobacter lovaniensis</i>	97.2	97.4	97.1	97.1	100	97.6	97	97.3	97.7	97.6	99.4	97.6	95.1	96.6	96.5
<i>Acetobacter orientalis</i>	97.8	99	97.4	98.3	97.6	100	98.4	96.8	97.1	97.1	97.7	98.7	95.2	96	95.9
<i>Acetobacter orleanensis</i>	97.8	98.1	97.5	98.3	97	98.4	100	95.9	96.5	96.2	96.9	98.7	95.1	95.8	95.7
<i>Acetobacter pasteurianus</i>	96.8	96.8	96.3	96.4	97.3	96.8	95.9	100	97.5	99.4	97.7	96.4	94.7	95.2	95
<i>Acetobacter peroxydans</i>	96.6	96.8	97.1	96.7	97.7	97.1	96.5	97.5	100	97.9	97.8	97	95.2	95.8	95.6
<i>Acetobacter pomorum</i>	97.3	97.2	96.6	96.7	97.6	97.1	96.2	99.4	97.9	100	97.9	96.7	95	95.7	95.5
<i>Acetobacter syzygii</i>	97.5	97.6	97.3	97.3	99.4	97.7	96.9	97.7	97.8	97.9	100	97.7	95.2	95.7	95.5
<i>Acetobacter tropicalis</i>	97.9	98.4	97.4	98.5	97.6	98.7	98.7	96.4	97	96.7	97.7	100	95.1	96.1	95.9
<i>Acidomonas methanohila</i>	95.2	94.7	94.9	94.7	95.1	95.2	95.1	94.7	95.2	95	95.2	95.1	100	96.2	96
<i>Asaia bogorensis</i>	96.5	95.7	96.6	95.7	96.6	96	95.8	95.2	95.8	95.7	96.4	96.1	96.2	96.2	96
<i>Asaia stamensis</i>	96.3	95.5	96.4	95.7	96.5	95.9	95.7	95	95.6	95.5	96.2	95.9	96	99.8	100
<i>Gluconacetobacter liquefaciens</i>	95.6	95.9	95.8	95.6	96.2	96.1	95.6	95.5	95.5	95.7	96.7	96	96.6	96.6	96.4
<i>Gluconacetobacter azolophilus</i>	95.9	95	95.4	95	95.6	95.4	95.4	94.9	95	95	96.1	95.6	96	96.4	96.2
<i>Gluconacetobacter diazotrophicus</i>	95.8	95.3	95.4	95.1	95.7	95.3	95.2	95.3	95.2	95.4	96.3	95.3	96	96.2	96
<i>Gluconacetobacter entanii</i>	95.3	94.9	95.3	94.8	95.4	95	95.1	94.8	94.5	94.9	95.6	95.6	95.5	95.9	95.7
<i>Gluconacetobacter europaeus</i>	95.5	95.1	95.5	94.9	95.8	95.1	95.3	94.9	94.5	95.1	95.9	95.8	95.8	96.3	96.1
<i>Gluconacetobacter hanseni</i>	95	94.6	94.9	94.5	95	94.7	94.6	94.3	94.1	94.5	95.3	95.2	95.3	95.5	95.4
<i>Gluconacetobacter intermedius</i>	95.7	95.3	95.8	95.2	96.2	95.4	95.7	95.3	94.9	95.4	96.1	96	95.8	96.6	96.5
<i>Gluconacetobacter johannae</i>	95.4	94.9	95.4	94.8	95.6	95.1	95.4	95.1	95.2	95.3	96	95.4	95.9	96.3	96.1
<i>Gluconacetobacter oboediens</i>	95.7	95.3	95.8	95.2	96.2	95.4	95.7	95.3	94.9	95.3	96.1	96	95.8	96.6	96.5
<i>Gluconacetobacter sacchari</i>	95.7	95.7	26.2	95.6	96.1	96.1	95.7	95.1	95.7	95.3	96.4	95.6	95.5	96.2	96.3
<i>Gluconacetobacter xylinus</i>	95.3	95	95.4	94.8	95.7	95	95.1	94.6	94.4	94.7	95.7	95.6	95.5	96.2	96
<i>Gluconobacter oxydans</i>	95.8	94.9	95.9	95	95.5	95.6	95.3	94.9	95.9	95.3	95.4	95.5	95	95.7	95.8
<i>Gluconobacter asaii</i>	95.3	94.6	95.6	94.9	95.4	95.1	95.1	94.3	95.6	94.8	95.5	94.9	94.6	96.2	96.2
<i>Gluconobacter cerinus</i>	95.3	94.6	95.6	94.9	95.4	95.1	95.1	94.3	95.6	94.8	95.5	94.9	94.6	96.2	96.2
<i>Gluconobacter frateurii</i>	95.5	94.6	95.4	94.8	95.5	95.1	95.1	94.8	96.2	95.2	95.7	94.9	95.2	96.1	96

(continued)

TABLE BXII.α.13. (cont.)

		% 16S rRNA sequence similarity															
Species		<i>Gluconacetobacter liquefaciens</i>	<i>Gluconacetobacter azotocaptans</i>	<i>Gluconacetobacter diazotrophicus</i>	<i>Gluconacetobacter entanii</i>	<i>Gluconacetobacter europaeus</i>	<i>Gluconacetobacter hanseni</i>	<i>Gluconacetobacter intermedius</i>	<i>Gluconacetobacter johannae</i>	<i>Gluconacetobacter oboediens</i>	<i>Gluconacetobacter sacharii</i>	<i>Gluconacetobacter xylinus</i>	<i>Gluconobacter oxydans</i>	<i>Gluconobacter asaii</i>	<i>Gluconobacter cerinus</i>	<i>Gluconobacter frateurii</i>	
<i>Acetobacter aceti</i>		95.9	95.6	95.8	95.3	95.5	95	95.7	95.4	95.7	95.7	95.3	95.8	94.6	95.3	95.5	
<i>Acetobacter cibinongensis</i>		95.9	95	95.3	94.9	95.1	94.6	95.3	94.9	95.3	95.7	95	94.9	93.7	94.6	94.6	
<i>Acetobacter estunensis</i>		95.8	95.4	95.4	95.3	95.5	94.9	95.8	95.4	95.8	96.2	95.4	95.9	94.6	95.6	95.4	
<i>Acetobacter indonesiensis</i>		95.6	95	95.1	94.8	94.9	94.5	95.2	94.8	95.2	95.6	94.8	95	94.1	94.9	94.8	
<i>Acetobacter lovaniensis</i>		96.2	95.6	95.7	95.4	95.8	95	96.2	95.6	96.2	96.1	95.7	95.5	94.6	95.4	95.5	
<i>Acetobacter orientalis</i>		96.1	95.4	95.3	95	95.1	94.7	95.4	95.1	95.4	96.1	95	95.6	94.3	95.1	95.1	
<i>Acetobacter orleanensis</i>		95.6	95.4	95.2	95.1	95.3	94.6	95.7	95.4	95.7	95.7	95.1	95.3	94.2	95.1	95.1	
<i>Acetobacter pasteurianus</i>		95.5	94.9	95.3	94.8	94.9	94.3	95.3	95.1	95.3	95.1	94.6	94.9	93.6	94.3	94.8	
<i>Acetobacter peroxidans</i>		95.5	95	95.2	94.5	94.5	94.1	94.9	95.2	94.9	95.7	94.4	95.9	94.7	95.6	96.2	
<i>Acetobacter pomorum</i>		95.7	95	95.4	94.9	95.1	94.5	95.4	95.3	95.4	95.3	94.7	95.3	94	94.8	95.2	
<i>Acetobacter syzygii</i>		96.7	96.1	96.3	95.6	95.9	95.3	96.1	96	96.1	96.4	95.7	95.4	94.7	95.5	95.7	
<i>Acetobacter tropicalis</i>		96	95.6	95.3	95.6	95.8	95.2	96	95.4	96	96.1	95.6	95.5	94	94.9	94.9	
<i>Acidomonas methanolytica</i>		96.6	96	96	95.5	95.8	95.3	95.8	95.9	96.3	96.4	95.5	95	93.8	94.6	95.2	
<i>Asaia bogorensis</i>		96.6	96.4	96.2	96.2	96.3	95.5	96.6	96.3	96.6	96.5	96.2	95.7	95.2	96.2	96.1	
<i>Asaia siamensis</i>		96.4	96.2	96	95.7	96.1	95.4	96.5	96.1	96.5	96.3	96	95.8	95.3	96.2	96	
<i>Gluconacetobacter liquefaciens</i>		100	98.1	98.5	97.2	97.4	97	97.2	98	97.2	99.1	97.2	94.3	93.8	94.7	94.9	
<i>Gluconacetobacter azotocaptans</i>		98.1	100	98.6	97.7	97.4	97.2	97	99.4	97	97.9	97.2	94.4	93.6	94.5	94.6	
<i>Gluconacetobacter diazotrophicus</i>		98.5	98.6	100	97.1	97	97	96.6	98.5	96.6	98.4	96.7	94.5	93.9	94.6	94.9	
<i>Gluconacetobacter entanii</i>		97.2	97.7	97.1	100	99	99.3	98.6	97.6	98.6	97	98.8	94.6	93.4	94.2	94.6	
<i>Gluconacetobacter europaeus</i>		97.4	97.4	97	99	100	98.6	99.5	97.3	99.5	97.1	99.6	94.8	93.6	94.6	94.9	
<i>Gluconacetobacter hanseni</i>		97	97.2	97	99.3	98.6	100	100	97	98.2	96.8	99.2	94.4	93.2	94	94.2	
<i>Gluconacetobacter intermedius</i>		97.2	97	96.6	98.6	99.5	98.2	100	97	100	97	99.2	95.3	93.9	94.9	95.3	
<i>Gluconacetobacter johannae</i>		98	99.4	98.5	97.6	97.3	97.1	97	100	97	97.9	97.2	94.4	93.6	94.5	94.6	
<i>Gluconacetobacter oboediens</i>		97.2	97	96.6	98.6	99.5	98.2	100	97	100	97	99.2	95.3	93.9	94.9	95.3	
<i>Gluconacetobacter sacharii</i>		99.1	97.9	98.4	97	97.1	96.8	97	97.9	97	100	97	94.4	93.6	94.4	94.7	
<i>Gluconacetobacter xylinus</i>		97.2	97.2	96.7	98.8	99.6	98.3	99.2	97.2	99.2	97	100	100	97	97.8	98	
<i>Gluconobacter asaii</i>		94.3	94.4	94.3	94.6	94.8	94.4	95.3	94.5	95.3	94.4	94.6	97.9	100	100	98.8	
<i>Gluconobacter cerinus</i>		94.7	94.5	94.6	94.2	94.6	94	94.9	94.5	94.9	94.4	94.4	97.9	100	100	98.8	
<i>Gluconobacter frateurii</i>		94.7	94.5	94.6	94.2	94.6	94	94.9	94.5	94.9	94.4	94.4	97.9	100	100	98.8	
<i>Gluconobacter frateurii</i>		94.9	94.6	94.9	94.6	94.9	94.2	95.3	94.6	95.3	94.8	94.7	98	98	98.8	100	

submerged fermentations carried out in acetators and trickle generators (Teuber et al., 1987b; Mariette et al., 1991). Plasmid profile analysis showed that acetators (suspensions of *Gluconacetobacter europaeus*) harbored only one dominant strain, whereas trickle generators (with a microflora in the form of biofilms on mechanical supports such as wooden chips) demonstrated a highly complex strain composition with spirit as substrate (Sievers and Teuber, 1995). Industrial submerged vinegar fermentations are initiated by inoculation with "seed vinegar", a microbiologically undefined fermentation broth from previous fermentations. The lack of defined pure starter cultures is due to the problems in the isolation, culture maintenance, determination of viable counts, and strain preservation of *Gluconacetobacter europaeus* strains responsible for high acid (up to 17% acetic acid) production. Sokollek and Hammes (1997) produced a starter culture of an acetic acid bacterium isolated from an industrial acetator with red wine as substrate for vinegar fermentation. The organism required no acetic acid for growth and is classified as *Gluconacetobacter oboediens* (Sokollek et al., 1998b). The frozen and lyophilized strain LTH 2460 was turned into a starter preparation by revitalization steps and stepwise adjustment of the growing culture to the acetic acid and ethanol composition of the medium used in industrial fermentation (Sokollek and Hammes, 1997). The use of a starter culture leads from spontaneous fermentation to a microbiologically controlled fermentation in vinegar production and reduces the time to start the acetator. RAPD (random amplified polymorphic DNA) analysis with a suitable primer (AGCGGGCGTA or CGCGTGCCCA or GTGGTGGTGGTGGTG) for the PCR was used for characterization of genotypically different strains from spirit vinegar fermentations (Trček et al., 1997). On a strain/species level, electrophoretic protein profiles (Kerstens and De Ley, 1975) of total cell protein extracts and their computerized evaluation are very useful for the definition of different phenons within the species *Gluconacetobacter europaeus* and *Gluconacetobacter intermedius* (Sievers and Teuber, 1995; Boesch et al., 1998). Numerical analysis of protein gel electrophoregrams of a wide variety of *Acetobacter* strains has been used for classificatory changes at species level within the genus *Gluconacetobacter* (Gosselé et al., 1983b). Electrophoretic mobilities of metabolic enzymes (multilocus enzyme electrophoresis) have been used to measure genotypic diversity of natural populations (Selander et al., 1986). This technique was applied to *Gluconacetobacter diazotrophicus* strains isolated from sucrose-rich host plants and mealy bugs to determine their degree of genetic relationships (Caballero-Mellado and Martínez-Romero, 1994; Caballero-Mellado et al., 1995). Nitrogen-fixing *Acetobacter* (*Gluconacetobacter*) strains isolated from a new host plant *Eleusine coracana* (finger millet) were characterized upon RAPD profiles grouping into two genetically related clusters (Loganathan et al., 1999). Pulsed-field gel electrophoresis with *Xba*I as restriction enzyme (electrophoresis for 24.5 h at 200 V in 1% agarose with a pulse time ramped from 5 to 40 s) is an appropriate tool for differentiation among strains of acetic acid bacteria.

Enrichment and isolation procedures A standard medium for enrichment and isolation of acetic acid bacteria, with the exception of *Gluconacetobacter europaeus* and *Gluconacetobacter entanii*, contains (per liter of distilled water) yeast extract, 5.0 g; peptone, 3.0 g; D-glucose, 0.5 g; ethanol (99.8%), 15 ml; cycloheximide, 0.1 g; and 12 g agar. The enrichment medium for the isolation of acetic acid bacteria, especially for *Asaia*, whose growth

is inhibited by 0.35% acetic acid, is composed of (per liter of distilled water): D-sorbitol, 20 g; peptone, 5 g; yeast extract, 3 g; and cycloheximide, 0.1g; adjusted to pH 3.5 with hydrochloric acid (Yamada et al., 2000).

Differentiation of acetic acid bacteria from other genera

Acetic acid bacteria (with the exception of *Asaia*) oxidize ethanol aerobically to acetic acid. Acetic acid is accumulated in the medium. After the complete oxidation of ethanol, *Acetobacter*, *Gluconacetobacter*, and *Acidomonas* oxidize acetic acid further to CO₂ and H₂O. This phenomenon provides a rapid phenotypic differentiation of the overoxidation capacity by use of the chalk-ethanol test of Carr and Passmore (1979). All species of acetic acid bacteria with the exception of *Gluconacetobacter europaeus*, *Gluconacetobacter entanii*, *Asaia bogorensis*, and *Asaia siamensis* grow well to moderately on the modified medium.² The acetic acid that is initially formed dissolves the calcium carbonate; then further oxidation of the acetic acid by *Acetobacter* and *Gluconacetobacter* leads gradually to a return of the chalk. This medium is used for isolation of acetic acid bacteria from flowers and fruits. A final concentration of 0.01% cycloheximide (stock-solution dissolved in ethanol) is used to prevent growth of most yeast cells.

Frateruia resembles *Acetobacter*, *Gluconacetobacter*, *Acidomonas*, and *Gluconobacter* in its ability to oxidize ethanol to acetic acid. *Frateruia* is not able to overoxidize acetate, but, in contrast to *Gluconobacter* oxidizes lactate to CO₂ and H₂O. *Frateruia* contains ubiquinone of the Q-8 type. Colonies of *Acidiphilium* are light brown, pale pink, red, or violet due to the presence of carotinoids and bacteriochlorophyll *a*. Some strains of *Acidiphilium* produce no bacteriochlorophyll *a*. Growth of *Acidiphilium* is inhibited in contrast to acetic acid bacteria in the presence of 0.25 mM acetate. *Acidiphilium acidophilum* (*Thiobacillus acidophilus*) can be differentiated from acetic acid bacteria by its ability to grow in mineral medium at pH values of 2–4 in the presence of H₂SO₄. *Acidocella* can be differentiated from other genera by its ability to use diaminobutane, 4-aminobutyrate, and 5-aminovaleate as carbon and energy source. *Rhodospila globiformis* grows phototrophically under anaerobic conditions. *R. globiformis* is able to grow photoheterotrophically using ethanol as electron donor and carbon source. The cell shape of *R. globiformis* is spherical. *Roseococcus thiosulfatophilus* contains bacteriochlorophyll *a* and is obligately aerobic. *R. thiosulfatophilus* can be differentiated from acetic acid bacteria by its formation of pink cocci, its oxidation of thiosulfate to sulfate, and its inability to use ethanol, D-mannitol, and D-fructose as carbon sources. *Ketogulonigenium vulgare* is oxidase positive and, in contrast to acetic acid bacteria, not able to grow at pH 4.5. The truncated stem-loop structure in the 16S RNA at position 1241–1296 in the *E. coli* numbering system distinguishes *Ketogulonigenium vulgare* from acetic acid bacteria and some other members of the *Alphaproteobacteria* (Urbance et al., 2001).

In the following chapters, we describe the genera *Gluconacetobacter*, *Acetobacter*, *Acidomonas*, *Asaia*, and *Gluconobacter* of the family *Acetobacteraceae*, which differ in respect to genetic and physiological properties like 16S rRNA sequence similarity, phylogenetic positioning, DNA–DNA similarity, ubiquinone type, oxidation of acetic acid to carbon dioxide and water, and tolerance to acetic acid.

2. Medium for the chalk-ethanol test (per liter of distilled water): glucose, 0.5 g; yeast extract, 5.0 g; peptone, 3.0 g; calcium carbonate, 15.0 g; agar, 12.0 g; and ethanol (99.8%), 15 ml (sterilized by filtration and added after sterilization of the basal medium).

Genus I. Acetobacter Beijerinck 1898, 215^{AL}

MARTIN SIEVERS AND JEAN SWINGS

A.ce.to.bac'ter. L. n. *acetum* vinegar; M.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod;
M.L.masc. n. *Acetobacter* vinegar rod.

Cells **ellipsoidal to rod shaped**, straight or slightly curved, 0.6–0.9 × 1.0–4.0 µm, occurring singly, in pairs, or in chains. **Motile or nonmotile; if motile, the flagella are peritrichous**. Endospores are not formed. Gram negative. **Obligately aerobic**; metabolism is strictly respiratory with oxygen as the terminal electron acceptor. Never fermentative. Form pale colonies; most strains produce no pigments. A minority of strains produces brown water-soluble pigments or show pink colonies due to the production of porphyrins. Usually catalase positive. Oxidase negative. Absence of gelatin liquefaction, indole production, and H₂S formation. **Oxidizes ethanol to acetic acid. Acetate is oxidized to CO₂ and H₂O**. The best carbon sources for growth are ethanol, glycerol, and glucose. Some strains require *p*-aminobenzoic acid, niacin, thiamin, or pantothenic acid as growth factors. Neither lactose nor starch is hydrolyzed. No production of 2,5-diketo-D-gluconate from D-glucose. Chemoorganotrophs. Optimum temperature is 30°C. Some strains reduce nitrate to nitrite. **The pH optimum for growth is 4.0–6.0. Possesses ubiquinone of the Q-9 type as major quinone**. The predominant fatty acid in *Acetobacter* is the C_{18:1 ω7} straight-chain unsaturated acid. ***Acetobacter* species occur in flowers, fruits, palm wine, vinegar, kefir, and fermented foods and can cause infections in grape wine, sake, tequila, cocoa wine, cider, beer, and fermented meat.** *Acetobacter* is not known to have any pathogenic effect toward humans and animals.

The mol% G + C of the DNA is: 50.5–60.3.

Type species: Acetobacter aceti (Pasteur 1864) Beijerinck 1898 (*Mycoderma aceti* Pasteur 1864, 125; *Acetobacter aceti* subsp. *aceti* (Pasteur 1864) De Ley and Frateur 1974.)

FURTHER DESCRIPTIVE INFORMATION

Cultivation media *Acetobacter aceti*, *A. pasteurianus*, *A. pomorum*, *A. orleanensis*, *A. peroxydans*, and *A. estunensis* grow well on standard medium (MYP).¹ *Acetobacter indonesiensis* and *Acetobacter tropicalis* are not able to utilize D-mannitol and were cultivated on a basal medium containing glucose, glycerol, and ethanol. Acetic acid bacteria are mesophilic strains with optimal temperature for growth at 28–30°C. Thermotolerant *Acetobacter* spp. capable of producing acetic acid at temperatures of about 37°C have been described (Ohmori et al., 1980; Saeki et al., 1997; Lu et al., 1999).

MAINTENANCE PROCEDURES

Strains of *Acetobacter* can be maintained for 2 weeks at 4°C on agar media used for cultivation. *Acetobacter* strains can be frozen and kept at –75°C in the presence of 24% (v/v) glycerol.

1. MYP medium (g/l distilled water): D-mannitol, 25.0; yeast extract, 5.0; peptone, 3.0; agar, 12.0.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ACETOBACTER*

Characteristics of the species of the genus *Acetobacter* are given in Table BXII.α.14.

TABLE BXII.α.14. Characteristics of the species of the genus *Acetobacter*^a

Characteristic ^b	<i>A. aceti</i>	<i>A. cibinongensis</i>	<i>A. estunensis</i>	<i>A. indonesiensis</i>	<i>A. lovaniensis</i>	<i>A. orientalis</i>	<i>A. orleanensis</i>	<i>A. pasteurianus</i>	<i>A. peroxydans</i>	<i>A. pomorum</i>	<i>A. syzygii</i>	<i>A. tropicalis</i>
Catalase	+	+	+	+	+	+	+	(+)	–	+	+	+
Ketogenesis from glycerol	+	–	–	–	–	–	d	d	–	+	–	–
Production of acid from D-glucose	+	+	+	+	+	+	+	d	–	(+)	+	+
<i>Production from D-glucose of:</i>												
2-keto-D-gluconate	+	+	+	+	–	+	+	–	–	–	–	+
5-keto-D-gluconate	+	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction ^c	–	–	+	d	d	–	d	+	–	nd	–	+
Ubiquinone type	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9
Mol% G + C content of DNA	56.2–57.2	53.8–54.5	59.3–59.7	53.3–55.1	57–59	52.0–52.8	56.5–58.7	51.8–53	60.3	50.5	54.3–55.4	55.2–56.6

^aSymbols: +, 90% or more of the strains positive; (+), weakly positive reaction; d, 11–89% of the strains positive; –, 90% or more of the strains negative; (–), most of the strains negative; nd, not determined.

^bData from Sievers et al. (1992); Sokollek et al. (1998b); Lisdiyanti et al. (2001a).

^cNitrate reduction was tested from nitrate peptone water (per liter of distilled water, pH 7.0: peptone, 10 g; KNO₃, 2 g) (Franke et al., 1999).

List of species of the genus *Acetobacter*

1. ***Acetobacter aceti*** (Pasteur 1864) Beijerinck 1898^{AL} (*Mycoderma aceti* Pasteur 1864, 125; *Acetobacter aceti* subsp. *aceti* (Pasteur 1864) De Ley and Frateur 1974.)

a. ce' ti. L. n. *acetum* vinegar; L. gen. n. *aceti* of vinegar.

Cell morphology and colonial characteristics are as described for the genus. All strains are ketogenic toward glycerol and sorbitol and most strains toward D-mannitol. Both 2-keto- and 5-ketogluconic acids are synthesized from D-glucose. 2,5-Diketogluconic acid is not produced. All strains acidify ethanol, *n*-propanol, *n*-butanol, D-xylose, D-mannose, and D-glucose. Ethanol, glycerol, D-mannitol, Na-acetate, and Na-D,L-lactate are good carbon sources for growth. All strains require growth factors in the presence of D-mannitol. Most strains utilize L-alanine and L-proline as a source of nitrogen in the presence of D-mannitol (De Ley et al., 1984). The level of 16S rRNA sequence similarity is 96.6–98.0% between *A. aceti* DSM 3508 and the type strains of the other *Acetobacter* species.

The mol% G + C of the DNA is: 56.2–57.2 (T_m).

Type strain: ATCC 15973, DSM 3508, IMET 10732, JCM 7641, NCIB 8621.

GenBank accession number (16S rRNA): D30768, X74066.

2. ***Acetobacter cibinongensis*** Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002, 3^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001a, 130.)

ci. bi. non' gen. sis. N.L. adj. *cibinongensis* derived from Cibinong, West Java, Indonesia.

A. cibinongensis is closely related to *Acetobacter orientalis* sharing 99.1–99.3% 16S rRNA sequence similarity by DNA–DNA similarity values of 12–25%. Strains produce D-glucuronate and 2-keto-D-glucuronate from D-glucose. Acid is produced from D-glucose, ethanol, and *n*-propanol. Acid production from L-arabinose, D-xylose, D-galactose, and D-mannose is strain dependent. *A. cibinongensis* does not produce acid from D-fructose, L-sorbose, D-arabinose, D-mannitol, D-sorbitol, glycerol, and lactose. Weakly positive for growth at 42°C, at pH 3.0 and pH 9.0, in the presence of 20% glucose, and in the presence of 10% ethanol (Lisdiyanti et al., 2001a). The level of 16S rRNA sequence similarity is 96.8–99.0% between *A. cibinongensis* IFO 16605 and the type strains of the other *Acetobacter* species. *A. cibinongensis* was isolated from fruit (mountain soursoy) and curd of tufu in Indonesia.

The mol% G + C of the DNA is: 53.8–54.5 (T_m).

Type strain: IFO 16605, JCM 11196, NRIC 0482.

GenBank accession number (16S rRNA): AB052710.

3. ***Acetobacter estunensis*** (Carr 1958) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001b, 263^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2000, 162) *Acetobacter pasteurianus* subsp. *estunensis* (Carr 1958) De Ley and Frateur 1974, 278; “*Acetobacter estunenses*” (sic) Carr 1958, 157.

es. tun. en' sis. M.L. adj. *estunensis* probably named after the old English name of the village Long Ashton, where J.G. Carr isolated the type strain.

A. estunensis was described by Carr (1958) as “*Acetobacter estunense*”. The name, “*A. estunense*”, implies that this or-

ganism is distinct from any species described by Frateur (Carr, 1958). *A. estunensis* oxidizes glucose to gluconate; 2-ketogluconate is produced but not 5-ketogluconate. Acid is produced from D-mannose and D-xylose but not from D-galactose, D-arabinose, L-arabinose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, or lactose. *A. estunensis* IFO 13751 contains 89.2% ubiquinone of the Q-9 type and 10.8% of the Q-8 type. *A. estunensis* IFO 13751 was isolated from cider. *A. estunensis* produced pellicles, which are not composed of cellulose since they dissolved after being boiled in 5% NaOH (Lisdiyanti et al., 2000). *A. estunensis* IFO 13751 showed 16S rRNA sequence similarity values of 96.3–97.9% to the type strains of other *Acetobacter* species.

The mol% G + C of the DNA is: 59.3–59.7 (T_m).

Type strain: ATCC 23753, DSM 4493, IFO 13751, LMG 1626, NCIMB 8935.

GenBank accession number (16S rRNA): AB032349.

4. ***Acetobacter indonesiensis*** Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001b, 263^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2000, 162.)

in. do. ne. si. en' sis. M.L. adj. *indonesiensis* referring to Indonesia where most strains studied were isolated.

A. indonesiensis cells are rod shaped, 1.8–2.0 × 0.8–1.0 μm, occurring singly, in pairs, or in chains. Colonies are circular, convex, glistening, and nonpigmented on basal medium containing 1.0% glucose, 1.0% glycerol, 1.0% ethanol, 1.0% peptone, 0.5% yeast extract, and 1.5% agar (Lisdiyanti et al., 2000). No growth on D-mannitol. Oxidizes glucose to gluconate; 2-ketogluconate is produced but not 5-ketogluconate. Does not produce acid from D-arabinose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, or lactose. Acid production from D-mannose, D-galactose, L-arabinose, and D-xylose is strain dependent. No ketogenesis from glycerol. *A. indonesiensis* IFO 16471 contains 86% ubiquinone of the Q-9 type and 14% of the Q-10 type (Lisdiyanti et al., 2000). The level of 16S rRNA sequence similarity is 96.4–98.6% between *A. indonesiensis* NRIC 0313 and the type strains of other *Acetobacter* species. *A. indonesiensis* was isolated from Indonesian fruits like banana, papaya, zizak, and mango. *A. indonesiensis* IFO 16471 was obtained from the fruit of zizak (*Annona muricata*).

The mol% G + C of the DNA is: 53.3–55.1 (T_m).

Type strain: IFO 16471, NRIC 0313.

GenBank accession number (16S rRNA): AB032356.

5. ***Acetobacter lovaniensis*** (Frateur 1950) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001b, 263^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2000, 162) (*Acetobacter pasteurianus* subsp. *lovaniensis* (Frateur 1950) De Ley and Frateur 1974, 278; “*Acetobacter lovaniense*” (sic) Frateur 1950, 336.)
- lo. va. ni. en' sis.* M.L. adj. *lovaniensis* referring to the city Louvain (Lovanium), in Belgium, where the type strain was isolated and studied by J. Frateur.

Acetobacter lovaniensis includes *A. pasteurianus* IFO 13753 and IFO 3284, *A. aceti* AJ 2913 and AJ 2914, and several Indonesian isolates from fruits (palm seed and starfruit) and fermented foods (nata de coco, moromi soya, palm

wine, and pickle) (Lisdiyanti et al., 2000). *A. lovaniensis* oxidizes glucose to gluconate. 2-ketogluconate and 5-ketogluconate are not produced. Does not produce acid from D-fructose, L-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, or lactose. Acid production from D-mannose, D-galactose, L-arabinose, and D-xylose is strain dependent. *A. lovaniensis* IFO 13753 contains 84.9% ubiquinone of the Q-9 type, 8.6% of the Q-8 type, and 6.5% of the Q-10 type (Lisdiyanti et al., 2000). The level of 16S rRNA sequence similarity is 97.0–99.4% between *A. lovaniensis* IFO 13753 and the type strains of other *Acetobacter* species.

The mol% G + C of the DNA is: 57–59 (T_m).

Type strain: ATCC 12875, DSM 4491, IFO 13753, LMG 1579, NCIMB 8620.

GenBank accession number (16S rRNA): AB032351.

6. ***Acetobacter orientalis*** Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002, 3^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001a, 130.) *ori.en'ta.lis*. M.L. adj. *orientalis* “oriental”, referring to the region where the strains were isolated.

A. orientalis cells are rod shaped, 2.0–3.0 × 0.6–0.8 µm. Colonies are circular, convex, glistening, and nonpigmented on basal medium pH 6.8 containing 1.0% glucose, 1.0% glycerol, 1.0% ethanol, 1.0% peptone, 0.5% yeast extract, and 1.5% agar (Lisdiyanti et al., 2001a). Strains produce D-gluconate and 2-keto-D-gluconate from D-glucose. Acid is produced from D-glucose, ethanol, and *n*-propanol. Acid production from L-arabinose, D-xylose, D-galactose, and D-mannose is strain dependent. *A. tropicalis* does not produce acid from D-fructose, L-sorbose, D-arabinose, D-mannitol, D-sorbitol, glycerol, and lactose. Weakly positive for growth at 42°C, at pH 3.0 and pH 9.0, in the presence of 20% glucose, and in the presence of 10% ethanol (Lisdiyanti et al., 2001a). *Acetobacter orientalis* was isolated from canna flower, fruits (starfruit and coconut), and fermented foods (curd of tofu and tempeh) in Indonesia. The level of 16S rRNA sequence similarity is 96.8–99.0% between *A. orientalis* IFO 16606 and the type strains of the other *Acetobacter* species.

The mol% G + C of the DNA is: 52–52.8 (T_m).

Type strain: IFO 16606, JCM 11195, NRIC 0481.

GenBank accession number (16S rRNA): AB052706.

7. ***Acetobacter orleanensis*** (Henneberg 1906) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001b, 263^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2000, 161) (*Acetobacter aceti* subsp. *orleanensis* (Henneberg 1906) De Ley and Frateur 1974, 278; “*Bacterium orleanense*” Henneberg 1906, 106.) *orle.an.en'sis*. M.L. adj. *orleanensis* referring to the city Orleans and the “Orleans method” for vinegar production.

A. orleanensis includes strains of *A. pasteurianus* IFO 13752, IFO 3170, and IFO 3223, *Gluconacetobacter hansenii* IFO 3296, and six Indonesian isolates from fruits (guava and sapodilla) and fermented foods (nata de coco) (Lisdiyanti et al., 2000). *A. orleanensis* oxidizes glucose to gluconate; 2-ketogluconate is produced but not 5-ketogluconate. Does not produce acid from D-arabinose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, or lactose. Acid production from D-mannose, D-galactose, L-arabinose,

and D-xylose is strain dependent. *A. orleanensis* IFO 13752 contains 81.0% ubiquinone of the Q-9 type, 14.2% of the Q-8 type, and 4.8% of the Q-10 type. The level of 16S rRNA sequence similarity is 95.9–98.7% between *A. orleanensis* IFO 13752 and the type strains of other *Acetobacter* species.

The mol% G + C of the DNA is: 56.5–58.7 (T_m).

Type strain: ATCC 12876, DSM4492, IFO 13752, LMG 1583, NCIMB 8622.

GenBank accession number (16S rRNA): AB032350.

8. ***Acetobacter pasteurianus*** (Hansen 1879) Beijerinck 1916, 1199^{AL} (*Mycoderma pasteurianum* Hansen 1879, 230; *Acetobacter pasteurianum* (sic) Beijerinck 1916, 1199; *Acetobacter pasteurianus* subsp. *ascendens* (Henneberg 1898) De Ley and Frateur 1974, 278; *Acetobacter pasteurianus* subsp. *paradoxus* (Frateur 1950) De Ley and Frateur 1974, 278.) *pas.teur.i.a' nus*. M.L. adj. *pasteurianus* of Pasteur; named after Louis Pasteur (1822–1895), French chemist and bacteriologist.

The cell morphology and colonial characteristics are as described for the genus. Not all strains are able to grow on GYC medium (5% D-glucose, 1% yeast extract, 3% CaCO₃, 1.5% agar). Most strains are negative for ketogenesis from glycerol and formation of 5-ketogluconic acid from D-glucose. Some strains are weakly catalase positive. Most strains produce acid from ethanol, *n*-propanol, and *n*-butanol, but not always from D-glucose. All strains require growth factors in the presence of D-mannitol (De Ley et al., 1984). *A. pasteurianus* was found in different kefir grains as milk adapted biotype at numbers varying between 3 × 10³ and 3 × 10⁶ CFU/g (Häfliger et al., 1991a, b). *A. pasteurianus* growing in kefir utilizes L-alanine and L-proline as nitrogen and carbon sources (Häfliger et al., 1991b). The level of 16S rRNA sequence similarity is 95.9–99.4% between *A. pasteurianus* DSM 3509 and the type strains of the other *Acetobacter* species.

The mol% G + C of the DNA is: 51.8–53 (T_m).

Type strain: ATCC 33445, DSM 3509, IMET 10733, LMD 22.1.

GenBank accession number (16S rRNA): X71863.

9. ***Acetobacter peroxydans*** Visser't Hooft 1925, 225^{VP} *per.ox'y.dans*. L. pref. *per* very; M.L. part. adj. *oxydans* acid-giving, oxidizing; M.L. part. adj. *peroxydans* strongly oxidizing.

Strain IFO 13755 is the type species and was isolated from ditch water. *A. peroxydans* IFO 13755 oxidizes ethanol, acetate, and lactate but does not produce acid from different carbon sources (D-glucose, D-mannose, D-galactose, D-arabinose, L-arabinose, D-xylose, D-fructose, DL-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, lactose). Does not produce 2-ketogluconate or 5-ketogluconate. Catalase negative. Peroxidase positive. The level of DNA relatedness of *A. peroxydans* IFO 13755 with other strains of *Acetobacter* is above 46%. The level of 16S rRNA sequence similarity is 96.5–97.9% between *A. peroxydans* IFO 13755 and the type strains of other *Acetobacter* species.

The mol% G + C of the DNA is: 60.3 (T_m).

Type strain: IFO 13755, ATCC 12874, LMG 1635, NCIMB 8618.

GenBank accession number (16S rRNA): AB032352.

10. **Acetobacter pomorum** Sokollek, Hertel and Hammes 1998b, 940^{VP}
po.mo'rum. L. n. *pomum* fruit; L. gen. pl. n. *pomorum* of the fruits.

Cells are $0.8\text{--}1.2 \times 1.3\text{--}1.6 \mu\text{m}$, nonmotile, and occur mainly in pairs. Colonies are round, regular, convex, soft to liquid, glossy, and beige with a diameter of $0.8\text{--}1.5 \text{ mm}$ on RAE agar (1a/2e). Plates of RAE agar are prepared by using the double layer technique as described by Entani et al. (1985) with 0.5% agar in the bottom layer and 1% agar in the top layer. RAE agar (1a/2e) is composed of 4% glucose, 1% yeast extract, 1% peptone, 0.338% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15% citric acid $\cdot \text{H}_2\text{O}$, 1% (v/v) acetic acid, and 2% (v/v) ethanol, in both agar layers (Sokollek and Hammes, 1997). Cells grow on 3% (v/v) ethanol in the presence of acetic acid levels as high as 4% (w/v). Growth occurs in the presence of 30% (w/v) glucose with a weak gluconic acid formation ($<10 \text{ g/l}$). 5- and 2-ketogluconic acid are not formed from glucose. Carbon sources include methanol, ethanol, *n*-propanol, D-glucose, D-fructose, D-maltose, D-ribose, D-xylose, D-sorbitol, D-mannitol, and glycerol. No growth occurs on sucrose, L-lactate, and D-gluconate. Dihydroxyacetone is formed from glycerol. Cellulose is not synthesized. *A. pomorum* DSM 11825 was isolated from a submerged cider vinegar fermentation. The level of 16S rRNA sequence similarity is 96.2–99.4% between *A. pomorum* DSM 11825 and the type strains of the other *Acetobacter* species.

The mol% G + C of the DNA is: 50.5 (T_m).

Type strain: DSM 11825, LTH 2458.

GenBank accession number (16S rRNA): AJ001632.

11. **Acetobacter syzygii** Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002, 3^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001a, 129.)

sy.zy'gi.i. L. gen. *syzygii* derived from N.L. neut. n. *syzygium* referring to the name of the fruit of the Malay rose apple (*Syzygium malaccense*) from which the type strain was isolated.

A. syzygii IFO 16604 is closely related to *A. lovaniensis*

IFO 13753 sharing 99.4% 16S rRNA sequence similarity by DNA–DNA similarity value of 38%. Strains of *A. syzygii* produce acids from D-glucose, ethanol, and *n*-propanol. 2 keto-D-gluconate is not formed. Acid production from L-arabinose, D-xylose, and D-mannose is strain dependent. Weakly positive for growth at 42°C, at pH 3.0 and pH 9.0, in the presence of 20% glucose, and in the presence of 10% ethanol (Lisdiyanti et al., 2001a). The level of 16S rRNA sequence similarity is 96.9–99.4% between *A. syzygii* IFO 16604 and the type strains of the other *Acetobacter* species.

The mol% G + C of the DNA is: 54.3–55.4 (T_m).

Type strain: IFO 16604, JCM 11197, NRIC 0483.

GenBank accession number (16S rRNA): AB052712.

12. **Acetobacter tropicalis** Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001b, 263^{VP} (Effective publication: Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2000, 163.)

tro.pi.ca'lis. M.L. adj. *tropicalis* referring to the tropical region where the strains were isolated.

A. tropicalis cells are rod shaped, $1.8\text{--}2.0 \times 0.5\text{--}0.7 \mu\text{m}$, occurring singly, in pairs, or in chains. Colonies are circular, convex, glistening, and nonpigmented on basal medium containing 1.0% glucose, 1.0% glycerol, 1.0% ethanol, 1.0% peptone, 0.5% yeast extract, and 1.5% agar (Lisdiyanti et al., 2000). No growth on D-mannitol. Oxidizes glucose to gluconate; 2-ketogluconate is produced but not 5-ketogluconate. Does not produce acid from D-arabinose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, glycerol, sucrose, or lactose. Acid production from D-mannose, D-galactose, L-arabinose, and D-xylose is positive to weak reaction. No ketogenesis from glycerol. The major quinone is Q-9 (86–100%). *A. tropicalis* IFO 16470 showed 16S rRNA sequence similarity values of 96.4–98.7% to the type strains of other *Acetobacter* species. *A. tropicalis* IFO 16470 was isolated from coconut (*Cocos nucifera*).

The mol% G + C of the DNA is: 55.2–56.6 (T_m).

Type strain: IFO 16470, NRIC 0312.

GenBank accession number (16S rRNA): AB032354, AB032355.

Genus II. Acidiphilium Harrison 1981, 331^{VP} emend. Kishimoto, Kosako, Wakao, Tano and Hiraishi 1995b, 90

AKIRA HIRAISHI AND JOHANNES F. IMHOFF

A.ci.di.phi'li.um. M.L. n. *acidum* an acid; Gr. adj. *philus* loving; M.L. neut. n. *Acidiphilium* acid lover.

Cells are straight rods, $0.3\text{--}1.2 \times 4.2 \mu\text{m}$, multiply by binary fission and exhibit a pleomorphic tendency at varied pH values and in the presence of different carbon sources. Motile by means of polar, subpolar, and lateral flagella. Do not form spores or capsules. Gram negative. **16S rRNA structures and signatures conform to the *Alphaproteobacteria*.** Cell suspensions and colonies are white to cream, yellow, pink, red, or brown. **Strictly aerobic, chemoorganotrophic and chemolithotrophic bacteria containing photosynthetic pigments**, categorized into the aerobic bacteriochlorophyll-containing bacteria. **Main photosynthetic pigments are zinc-chelated bacteriochlorophyll (Zn-BChl) *a* and the carotenoid spirilloxanthin.** Grow with simple organic compounds, such as sugars, as electron donors and carbon sources. **Growth**

is inhibited in the presence of 0.6% yeast extract and low concentrations of acetate (0.25 mM) and lactate (2 mM). One species is a facultative chemolithotroph utilizing elemental sulfur as electron donor. Fe^{2+} does not serve as the electron donor for chemolithotrophic growth but has stimulatory effects on heterotrophic growth. Catalase is positive and oxidase negative or weakly positive. Mesophilic and **obligately acidophilic bacteria** growing in the pH range of 2.0–5.9 (but not at pH 6.1 and above). **Straight-chain monounsaturated $\text{C}_{18:1}$ acid is the major component of cellular fatty acids.** Ubiquinones with ten isoprene (Q-10) units are present.

The mol% G + C of the DNA is: 62.9–68.1.

Type species: ***Acidiphilium cryptum*** Harrison 1981, 331.

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic analyses based on 16S rDNA sequence information have shown that *Acidiphilium* species form a major cluster in the *Alphaproteobacteria*, together with members of the acidophilic chemotrophic bacteria of the genera, *Acidocella*, *Acetobacter*, and *Gluconobacter*, and the acidophilic anoxygenic phototrophic bacterium *Rhodospila* (Sievers et al., 1994a; Kishimoto et al., 1995b). This cluster is deeply branched off from the *Alphaproteobacteria*. (See Fig. 1 p. 136 in the essay "Aerobic Bacteria Containing Bacteriochlorophyll and Belonging to the *Alphaproteobacteria*", Volume 2, Part A.)

Cells of *Acidiphilium* species are straight or slightly curved rods with rounded ends (Figs. BXII.α.18 and BXII.α.19). They vary in size from $0.4\text{--}0.8 \times 1.0\text{--}3.0 \mu\text{m}$ under optimal growth conditions, but cell size and shape are influenced by the kind and concentration of carbon source and by physico-chemical growth conditions (the range of cell size is shown in the generic description). Swollen cells and filaments ($>10 \mu\text{m}$ long) frequently occur when growing in nutrient-rich media, and this tendency is most pronounced in *Acidiphilium rubrum*. The swollen cells contain discrete, refractive, sudanophilic granules that are possibly poly- β -hydroxybutyrate granules. Electron microscopy of negatively stained cells shows that motile cells have single polar or subpolar flagellum (Fig. BXII.α.20). Two lateral flagella are occasionally found in *Acidiphilium cryptum* (Harrison, 1989) and *Acidiphilium multivorum*. Thin-section electron microscopy shows that any type of intracytoplasmic membrane systems is absent in the cells (Matsuzawa et al., 2000) (Fig. BXII.α.21). Polyphosphate granules as well as poly- β -hydroxybutyrate granules are frequently observed.

Acidiphilium species are strictly aerobic, chemoorganoheterotrophic bacteria (a single species is chemolithotrophic) that require high acidity for growth. They grow in the pH range of 2.5–5.9, some as low as 1.5–2.0, but not at pH 6.1 and above. Growth rates vary remarkably among the species. The type species *A. cryptum* and its closest relatives, *A. multivorum* and *A. organovorum*, exhibit a doubling time of 3–9 h, whereas *A. acidophilum* and *A. rubrum* grow much more slowly with a doubling time of more than 10 h under optimal growth conditions. *Acidiphilium*

species use simple organic compounds as carbon and energy sources for growth. The following sugars and sugar alcohols are good substrates supporting growth of all species: L-arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-fructose, sucrose, glycerol, arabitol, and mannitol. *Acidiphilium* species utilize the pentose phosphate and Entner–Doudroff pathways for glucose metabolism and lack the Embden–Meyerhoff–Parnas pathway (Shuttleworth et al., 1985). None of the strains uses the following carbon sources: L-rhamnose, cellobiose, trehalose, melibiose, formate, butanol, acetate, propionate, butyrate, valerate, caproate, lactate, pyruvate, glycolate, oxalate, DL- β -hydroxybutyrate, benzoate, *p*-hydroxybenzoate, glycine, cysteine, and lysine. Growth of *Acidiphilium* species in glucose-mineral media is inhibited by low concentrations of acetate or lactate. In addition, succinate and fumarate inhibit growth of some species. Yeast extract at a low concentration of less than 0.1% (w/v) enhances growth of *Acidiphilium* species, but at 0.3% and above it has inhibitory effects on the growth (Harrison, 1981; Kishimoto et al., 1990). This is due to the effects of organic acids contained in the yeast extract (Kishimoto et al., 1990). Trypticase and peptone have similar inhibitory effects.

A. acidophilum is the only species that exhibits chemolithotrophic growth with reduced sulfur compounds as energy sources, but other species may have the ability to oxidize elemental sulfur to sulfate (Harrison, 1981). A number of studies have demonstrated sulfur-dependent chemolithotrophy of and sulfur oxidation by *A. acidophilum* (Guay and Silver, 1975; Harrison, 1981; Pronk et al., 1990; Meulenberg et al., 1992; Hiraishi et al., 1998).

All species are capable of growth on high concentrations of metals, such as 100 mM Fe^{2+} and 100 mM Al^{3+} . *A. rubrum* may accumulate Fe as magnetite (Itoh et al., 1998; Matsuzawa et al., 2000). All species also exhibit high resistance to heavy metals such as copper, nickel, and zinc (Mahapatra and Banerjee, 1996). However, neither Fe^{2+} nor other metal ions can serve as an electron donor for chemolithotrophic growth. However, the addition of ferrous ion stimulates heterotrophic growth significantly. Some strains of *Acidiphilium* species, including *A. cryptum* are capable of coupling the reduction of Fe(III) to the oxidation of a variety of substrates under aerobic or anaerobic conditions (Johnson and McGinness, 1991; Pronk and Johnson, 1992; Johnson, 1998; Küsel et al., 1999). They may co-respire oxygen and Fe(III) under oxic conditions.

A. multivorum has the ability to oxidize arsenite to arsenate, whereas all other species lack this property (Wakao et al., 1994). *A. multivorum* has a number of plasmids of different sizes, one of which is 56 kbp and encodes its arsenic resistance. The arsenic resistance (*ars*) operon from the *A. multivorum* plasmid was cloned and sequenced (Suzuki et al., 1998). This cluster contained five genes in the following order: *arsR*, *arsD*, *arsA*, *arsB*, *arsC*, and the deduced amino acid sequences of all the gene products are homologous to the amino acid sequences of the *ars* gene products of the *Escherichia coli* plasmids. The *ars* operon cloned from *A. multivorum* conferred resistance to arsenate and arsenite upon *E. coli*.

One of the most outstanding characteristics of *Acidiphilium* species is the production of photopigments with Zn-BChl *a* as the major component (for abbreviations for metal-substituted bacteriochlorophylls [BChls] see Takaichi et al., 1999). The chemical structure of Zn-BChl *a* isolated from *Acidiphilium* is the same as Mg-BChl *a* esterified with phytol, except the occurrence of Zn in place of Mg as the central metal (Wakao et al., 1996;

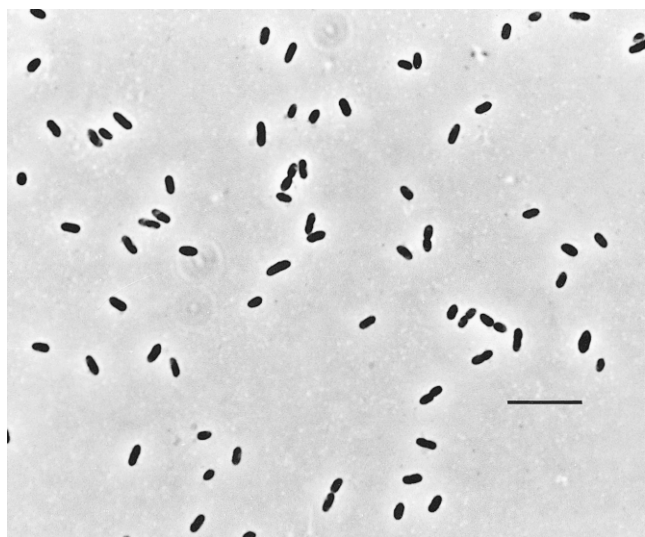


FIGURE BXII.α.18. Phase-contrast photomicrograph showing general cell morphology of *Acidiphilium cryptum*. Bar = 5 μm .

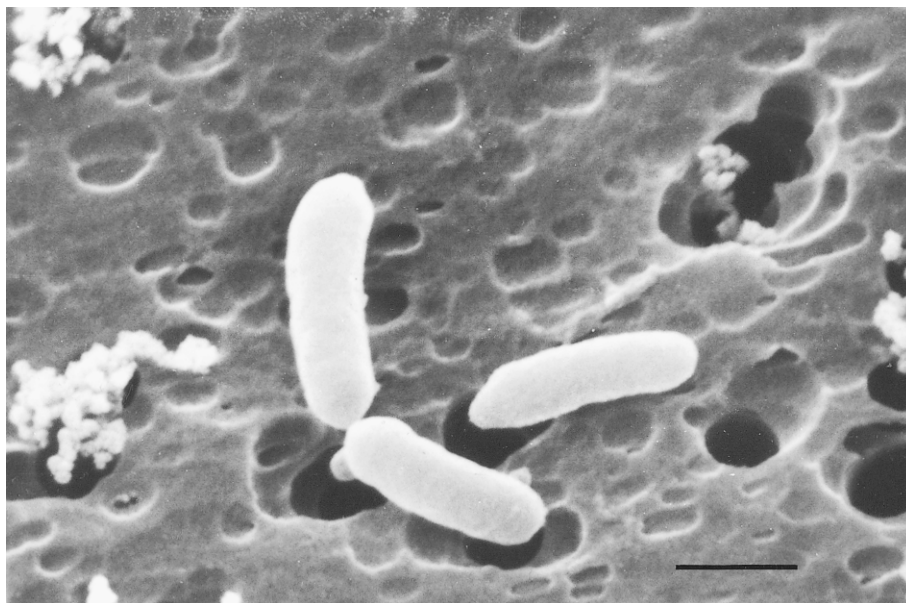


FIGURE BXII.α.19. Scanning electron photomicrograph showing general cell morphology of *Acidiphilium rubrum* ATCC 35905. Bar = 0.9 μm.



FIGURE BXII.α.20. Electron photomicrograph of a negatively stained cell of *Acidiphilium cryptum* ATCC 33463 having a subpolar flagellum. Bar = 0.5 μm. (Reprinted with permission from A. Hiraishi and K. Shimada, *Journal of General Applied Microbiology* 47: 161–180, 2001, ©Japan Society for Bioscience, Biotechnology, and Agrochemistry.)

Kobayashi et al., 1998). The dominant occurrence of Zn-BChl *a* is indicated by an absorption maximum at 763 nm in the acetone-methanol extract, which is blue-shifted by 7 nm compared to the absorption maximum of Mg-BChl *a* in the corresponding near infrared region. Among the *Acidiphilium* species so far estab-

lished, *A. rubrum* is most remarkable for Zn-BChl *a* production and contains 0.8–1.0 nmol of Zn-BChl *a* per mg dry weight of cells under optimal growth conditions. The cellular content of Zn-BChl *a* varies depending on environmental conditions. The production of Zn-BChl *a* occurs only under oxic-dark conditions,



FIGURE BXII.α.21. Thin-section electron photomicrograph of cells of *Acidiphilium rubrum* ATCC 35905. Bar = 0.5 μ m.

indicating the strong requirement of energy produced via aerobic respiratory electron transport for pigment production. Light inhibits photopigment production even under optimal growth conditions, as is the case in other genera of aerobic bacteriochlorophyll-containing bacteria. *Acidiphilium* species produce Mg-BChl *a* and bacteriopheophytin (BPhe) in addition to Zn-BChl *a*, but Zn-BChl *a* always predominates. The molar ratio of Zn-BChl *a*:Mg-BChl *a*:BPhe in the cells is relatively constant among the species; e.g., *A. rubrum* produces them at a molar ratio of 13:2:1 (Wakao et al., 1996; Hiraishi et al., 1998). In *A. rubrum*, a single set of magnesium chelatase homologs catalyzes the insertion of only Mg^{2+} into protoporphyrin IX to yield magnesium protoporphyrin IX monomethyl, and Zn-BChl *a* may be formed by a substitution of Zn^{2+} for Mg^{2+} at a step in the bacteriochlorophyll biosynthesis after formation of magnesium protoporphyrin IX monomethyl ester (Masuda et al., 1999).

Membrane preparations of *A. rubrum* have absorption maxima at 377, 486, 515, 549, 590, 792, and 864 nm. A much lower absorption maximum at around 800 nm compared to the maximum at 864 nm indicates that the cells contain light-harvesting complex I together with the photosynthetic reaction center but lack the peripheral light-harvesting complex LH II, like cells of the anoxygenic phototrophic bacterium *Rhodospirillum rubrum*. Spirilloxanthin is the sole carotenoid component of the antenna complex. Unlike other genera of aerobic bacteriochlorophyll-containing bacteria, such as *Erythrobacter* and *Porphyrobacter*, *Acidiphilium* contains no membrane-free polar carotenoids.

Photosynthetic activities were examined by light-induced absorption changes of the membrane-bound pigments of *A. rubrum* (Wakao et al., 1996). The light-induced difference spectrum showed absorption changes at around 800 and 850 nm, caused by activities of the monomer and special pair BChl molecules in the reaction center complex, respectively. The peak positions

assigned to the monomer and special pair BChls are blue-shifted by 7 and 25 nm, respectively, compared to the analogous positions found in *R. rubrum*. This suggests the involvement of Zn-BChl *a*, instead of Mg-BChl *a*, in the photochemical reaction. Although representative species of *Acidiphilium* have been shown to contain a fully active photochemical reaction center, they neither grow anaerobically in the light nor produce photopigments under anoxic or oxygen-limited conditions. The biological significance of the photosynthetic system in *Acidiphilium* has not been fully understood, but it has been shown that light enhances $^{14}CO_2$ incorporation into cells of *A. rubrum* under oxic conditions (Kishimoto et al., 1995a).

Nagashima et al. (1997b) have demonstrated that *Acidiphilium* species contain the *puf* operon, an assemblage of genes coding for α and β polypeptides of the light-harvesting complex LH I, the L and M subunits of photosynthetic reaction center proteins, and the membrane-bound cytochrome *c* subunit. The *puf* operon of *A. rubrum* consists of *pufB*, -*A*, -*L*, -*M*, and -*C* as found in general in anoxygenic phototrophic purple bacteria. A comparative analysis of deduced amino acid sequences showed that His L168, which is highly conserved in the L subunit in the anoxygenic phototrophic bacteria, is replaced by glutamic acid in *Acidiphilium*. This residue was suggested to locate closely to the special pair of BChl molecules and to be involved in the stabilization and function of Zn-BChl *a*, if the three dimensional structures of the *Blastochloris viridis* and *Rhodobacter sphaeroides* reaction centers were taken into account for comparison. A continuous stretch of amino acid sequences of the L and M subunits of *Acidiphilium* species is highly conserved, and a phylogenetic tree based on these amino acid sequences is basically consistent with the 16S rDNA sequence-based tree in the genealogical relationships between *Acidiphilium* and other phototrophic purple bacteria (Hiraishi et al., 1998).

Acidiphilium species contain high amounts (33–43% of the total lipids) of phosphorus-free lipoamino acids in their cell membranes. Two major lipoamino acids were isolated from *A. organovorum* and identified as being α -N-3-hydroxystearylornithinytaurine and α -N-3-hydroxystearylornithine, to which C_{18:1} fatty acid is linked by an ester-linkage (Kishimoto et al., 1993a). In addition to these lipids, bisphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine are present as the major phospholipids. Straight-chain mono-unsaturated C_{18:1} acid is the major component of whole-cell fatty acids, constituting of 40–90% of the total content (Kishimoto et al., 1993a; Wakao et al., 1994). A considerable amount of cyclopropane C_{19:0} acid (5–21%) is also present.

Acidiphilium species (including new isolates of acidophilic heterotrophic bacteria that most likely belong to this genus) have been isolated from acidic mineral environments, such as pyritic mine drainage, pyritic coal refuse, copper and uranium mine tailings, acidic hot springs, and acidic soil (Tuttle et al., 1968; Manning, 1975; Wichlacz and Unz, 1981; Wichlacz et al., 1986; Kishimoto and Tano, 1987; Harrison, 1989; Wakao et al., 1994; Banerjee et al., 1996; A. Hiraishi, unpublished data). Also, as several strains of *Acidiphilium* species were isolated or found as contaminants from cultures of the iron-oxidizing chemolithotroph *Thiobacillus ferrooxidans* (Guay and Silver, 1975; Harrison et al., 1980; Harrison, 1981, 1989; Johnson and Kelso, 1983; Lobos et al., 1986), they may aid leaching through the consumption of organic matter inhibitory to the growth and activity of *T. ferrooxidans*. Although most *Acidiphilium* species are obligately heterotrophic, their natural habitats seem to be oligotrophic environments with poor organic matter. The ability of *Acidiphilium* to perform photosynthesis with Zn-BChl *a* may be an advantage to grow and survive in such oligotrophic acidic environments. Zn-BChl *a* is much more stable under acidic conditions than Mg-BChl *a* and chlorophylls (Wakao et al., 1996). Therefore, it is likely that the capacity for photosynthesis with Zn-BChl *a* of *Acidiphilium* is a result of its adaptation to acidic mineral environments. In addition, the ability of *Acidiphilium* species to reduce Fe(III), using a variety of organic substrates indicates that they may be of ecological significance in the biogeochemical cycling of iron at oxic-anoxic interfaces in acidic environments.

ENRICHMENT AND ISOLATION PROCEDURES

A suitable medium for growth of *Acidiphilium* contains, per liter of distilled water: 2 g (NH₄)₂SO₄, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.05 g CaCl₂·2H₂O, and 1 ml trace element solution SL8 (Biebl and Pfennig, 1978), to which are added 0.1% glucose and 0.01% yeast extract (Difco Laboratories). The organic supplements are autoclaved separately. For solid media separately autoclaved 1.5% agar is added; alternatively, 0.6% gelatin gum can be used (Kishimoto and Tano, 1987). For isolation and enrichment of *Acidiphilium* from the environment, the medium should be adjusted to pH 2.0–2.5 to minimize growth of fungi and facultatively acidophilic bacteria. Although *Acidocella* strains are frequently recovered on isolation media, it is possible to differentiate colonies of *Acidiphilium* from those of *Acidocella* by pigmentation when the media are incubated for more than one week.

MAINTENANCE PROCEDURES

Cultures are well preserved in liquid nitrogen, by lyophilization, or at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *ACIDIPHILIUM* FROM OTHER GENERA

The genus *Acidiphilium* is separated from other genera of aerobic acidophilic heterotrophic bacteria by its phylogenetic position and its outstanding physiological and chemotaxonomic characteristics. Differential characteristics of *Acidiphilium* and phenotypically related genera are indicated in Table BXII.α.15.

TAXONOMIC COMMENTS

The genus *Acidiphilium* was first monotypic with *A. cryptum* as the type species (Harrison, 1983). Later, several species including *A. angustum*, *A. facilis*, *A. organovorum*, and *A. rubrum* (Lobos et al., 1986; Wichlacz et al., 1986) were identified as new members of this genus. A phylogenetic analysis based on 5S rRNA sequences showed a close relationship between *A. cryptum* and *Thiobacillus acidophilus* (Lane et al., 1985). Information about the phylogeny and photosynthetic features of *Acidiphilium* has increasingly been accumulated in the 1990s. Wakao et al. (1993) reported that some members of *Acidiphilium* including the type species *A. cryptum* produced BChls. This report was the first to demonstrate the occurrence of photosynthetic pigments in *Acidiphilium* species. Similar observations were reported by Kishimoto et al. (1995a). Phylogenetic analyses based on 16S rDNA sequences revealed that all species of *Acidiphilium* form a major phylogenetic cluster in the *Alphaproteobacteria* (Lane et al., 1992; Sievers et al., 1994a) and that the BChl-producing species were genetically distant from the non-BChl-producing ones (Kishimoto et al., 1995b). This finding led to the proposal to remove the non-BChl producing species (i.e., *Acidiphilium facilis* and *Acidiphilium aminolytica*) from the genus *Acidiphilium* and to transfer them into the new genus *Acidocella* (Kishimoto et al., 1995b). The emended description of *Acidiphilium* only includes aerobic BChl-containing bacteria.

Acidiphilium angustum and *A. rubrum* were described as distinct species by Wichlacz et al. (1986) based mainly on phenotypic data. However, these two species have been shown to be indistinguishable by 16S rDNA sequencing and genomic DNA–DNA hybridization (Wakao et al., 1994; Kishimoto et al., 1995a; Hiraishi et al., 1998; Table BXII.α.16) and in addition are phenotypically quite similar to each other. These data strongly suggest that the two species names are subjective synonyms. Here we propose to unite *A. angustum* and *A. rubrum* in a single species, retaining the name *A. rubrum*.

Thiobacillus acidophilus (Guay and Silver, 1975) was not included in the Approved List of Bacterial Names (Skerman et al., 1980). After reexamination of the characteristics of *Thiobacillus acidophilus* and confirmation that this bacterium was a facultative chemolithotroph growing equally with elemental sulfur or glucose as the sole energy source, Harrison (1983) revived this name. However, phylogenetic analyses based on small-subunit rRNA sequences showed that *Thiobacillus acidophilus* was more closely related to members of the genus *Acidiphilium* than to any other species of *Thiobacillus* (Lane et al., 1985, 1992; Kishimoto et al., 1995b). Therefore, based on the phylogenetic evidence and the finding that this bacterium contains Zn-BChl *a* and the *puf* genes, as the other *Acidiphilium* species, the transfer of *Thiobacillus acidophilus* to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov. was proposed (Hiraishi et al., 1998).

FURTHER READING

Harrison, Jr., A.P. 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. *Ann. Rev. Microbiol.* 38: 265–292.

TABLE BXII.α.15. Differential characteristics of the genus *Acidiphilium* and phenotypically related genera^a

Characteristic	<i>Acidiphilium</i>	<i>Acidocella</i>	<i>Acidobacterium</i>
Yellow-orange pigments	D	—	+
Pink-red pigments	D	—	—
Zn-BChl production	+	—	—
Growth inhibition by:			
Acetate (0.25 mM)	+	—	—
Lactate (2 mM)	+	—	—
Chemolithotrophic growth with sulfur	D	—	—
β-Galactosidase activity	—	—	+
Major fatty acid	C _{18:1}	C _{18:1}	iso-C _{15:0}
Major quinone	Q-10	Q-10	MK-8
Bisphosphatidylglycerol	+	—	+
Mol% G + C of DNA	63.2–67.5	58.7–64.4	60.8

^aSymbols: +, 90% or more of strains positive; —, 90% or more of strains negative; D, different reactions in different species; Q-10, ubiquinone-10; MK-8, menaquinone-8.

TABLE BXII.α.16. Inter- and intraspecies relatedness of genomic DNA in the genus *Acidiphilium*^a

Species	Level of hybridization (%)				
	<i>A. cryptum</i>	<i>A. acidophilum</i>	<i>A. angustum/A. rubrum</i>	<i>A. multivorum</i>	<i>A. organovorum</i>
<i>A. acidophilum</i>	80–100				
<i>A. angustum/A. rubrum</i>	18–22	71–100			
<i>A. cryptum</i>	76–100	8	11–26		
<i>A. multivorum</i>	43–63	10	10–29	83–100	
<i>A. organovorum</i>	64	10	9–26	53–56	100

^aData from Wakao et al. (1994) and Hiraishi et al. (1998).

Hiraishi, A. and S. Shimada. 2001. Aerobic anoxygenic photosynthetic bacteria with zinc-bacteriochlorophyll. *J. Gen. Appl. Microbiol.* 47: 161–180.

Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ACIDIPHILUM*

The differential characteristics of the species of *Acidiphilium* are indicated in Table BXII.α.17. Data on genomic DNA relatedness among the species are presented in Table BXII.α.16. Genetically, *Acidiphilium* species can be classified into two subgroups, one of which consists of *A. cryptum*, *A. multivorum*, and *A. organovorum* (*A. cryptum* subgroup) and one of which includes *A. acidophilum* and *A. rubrum* (*A. rubrum* subgroup). The levels of 16S rDNA sequence similarity and DNA–DNA hybridization between the two subgroups are 94–95% and 8–26%, respectively. Species differentiation in the former subgroup is problematic because of only small phenotypic and genetic differences among the species of this subgroup (Tables BXII.α.16 and BXII.α.17). It is impos-

sible to separate *A. cryptum*, *A. multivorum*, and *A. organovorum* by 16S rDNA sequencing (>99.5% interspecies similarity). In addition, genomic DNA–DNA reassociation levels among the three species are relatively high (43–63%). The diagnostic characteristics useful for the identification of the species include growth inhibition by different concentrations of glucose, yeast extract, and trypticase and carbon nutrition (fumarate, succinate, sorbitol, and inositol). However, since the available phenotypic information has been derived from studies of only limited numbers of strains, DNA–DNA hybridization assays are required for the ultimate identification at the species level.

List of species of the genus *Acidiphilium*

1. *Acidiphilium cryptum* Harrison 1981, 331^{VP} *cryp'tum*. Gr. adj. *kryptos* hidden.

Cells are straight rods, 0.3–0.6 × 0.6–1.5 μm under optimal growth conditions, occurring singly or in pairs. Motile by means of a single polar, or subpolar, flagellum or by two lateral flagella; some strains appear to be nonmotile. Colonies on agar media are circular (1–3 mm in diameter after one week of incubation), smooth, convex, slightly translucent, and white to cream, and turn pink to light brown after continued incubation. Aerobic, chemoorganoheterotrophic bacteria having a respiratory type of metabolism with oxygen as terminal electron acceptor. Some strains may grow with Fe(III) as the terminal electron acceptor under both oxic and anoxic conditions. Carbon sources utilized are listed in Table BXII.α.17. D-Glucose, D-fructose, and

some other sugars support good growth. Growth is inhibited by the presence of 1% glucose, 0.3% yeast extract, and 1% trypticase. Growth factors are required and this requirement is satisfied by 0.01% yeast extract. Ammonium salts are used as nitrogen source. Catalase is produced. Oxidase reaction is weakly positive or absent. Urease is negative. Arsenite oxidation is negative. Susceptible to ampicillin, chloramphenicol, rifampin, tetracycline, but resistant to josamycin, lincomycin, penicillin, and streptomycin. Optimal growth occurs at 30–37°C (range: 15–42°C) and at pH 3.0–3.5 (range: pH 2.0–5.9). No growth occurs at 42°C or with 3% NaCl. Habitat: Originally isolated from an iron-oxidizing culture of *Thiobacillus ferrooxidans* and found in strongly acidic environments, including mine water.

TABLE BXII.α.17. General and differential characteristics of the species of the genus *Acidiphilium*^{a,b}

Characteristic	<i>A. cryptum</i>	<i>A. acidophilum</i>	<i>A. angustum/A. rubrum</i>	<i>A. multivorum</i>	<i>A. organovororum</i>
Cell width (μm)	0.3–0.6	0.5–0.8	0.7–0.9	0.5–0.9	0.5–0.7
Motility	+	d	d	d	+
<i>Color of colonies:</i>					
Cream/pink/ light brown	+	+	–	+	+
Red/violet	–	–	+	–	–
Zn-BChl content	Low	Trace	High	Low	Trace
Growth factor required	+	–	+	+	+
<i>Chemolithotrophic growth:</i>					
Sulfur	–	+	–	–	–
Thiosulfate	–	+	–	–	–
Growth at 42°C	+	–	–	+	–
Growth with 3% NaCl	+	–	–	+	+
<i>Growth inhibition by:</i>					
1% Glucose	+	+	+	–	–
5% Glucose	+	+	+	–	+
0.3% Yeast extract	+	+	+	–	–
1% Trypticase	+	+	+	–	–
Arsenite oxidation	–	–	–	+	–
<i>Carbon source utilization:</i>					
L-Arabinose	+	+	+	+	+
D-Xylose	+	+	+	+	+
D-Ribose	+	+	+	+	+
L-Sorbose	nd	–	nd	+	nd
D-Fructose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Galactose	+	+	+	+	+
D-Mannose	d	+	+	+	+
D-Maltose	+	–	–	–	–
Sucrose	+	+	+	+	+
Lactose	+	–	–	–	–
Raffinose	+	–	–	+	nd
Melibiose	nd	nd	nd	+	nd
Mannitol	+	+	+	+	+
Sorbitol	+	nd	–	+	+
Glycerol	+	+	d	+	+
Inositol	–	nd	–	+	+
Methanol	–	+	–	+	–
Ethanol	d	+	d	+	–
Propanol	–	+	–	+	–
Pyruvate	–	–	–	–	–
Lactate	–	–	–	–	–
Acetate	–	–	–	–	–
Citrate	d	+	+	+	+
Oxoglutarate	d	–	d	+	–
Fumarate (2 mM)	–	–	–	+	+
Succinate (2 mM)	–	–	–	+	+
Malate	d	+	d	+	+
Malonate	nd	–	–	–	nd
Tartrate	+	–	nd	–	–
Gluconate	–	+	nd	+	+
Alanine	–	–	–	+	nd
Leucine	nd	–	nd	–	–
Isoleucine	nd	–	nd	–	–
Proline	nd	+	nd	+	+
Aspartate	nd	+	+	+	nd
Asparagine	+	nd	nd	+	nd
Glutamate	+	+	d	+	+
Arginine	+	nd	–	+	+
Lysine	–	nd	–	+	–
Histidine	nd	+	nd	nd	nd
Phenylalanine	nd	–	nd	+	+
Tryptophan	nd	–	nd	–	–
<i>Sensitivity to:</i>					
Penicillin	–	nd	nd	+	–
Streptomycin	–	nd	nd	+	–
Mol% G + C content of DNA	67.3–68.3	62.9–63.2	63.2–63.4	66.2–68.1	67.4

^aSymbols: +, 90% or more of strains positive; –, 90% or more of strains negative; d, 11–89% of strains positive; nd, not determined^bData from Wakao et al. (1994) and Hiraishi et al. (1998).

The mol% G + C of the DNA is: 68–70 (T_m), 69 (Bd), 67.3–67.5 (HPLC).

Type strain: Lhet2, ATCC 33463, DSM 2389.

GenBank accession number (16S rRNA): D30773.

2. **Acidiphilium acidophilum** (Harrison 1983) Hiraishi, Nagashima, Matsuura, Shimada, Takaichi, Wakao and Katayama 1998, 1396^{VP} (*Thiobacillus acidophilus* (Guay and Silver 1975) Harrison 1983.)

a.ci.do'phi.lum. L. adj. *acidus* sour; M.L. neut. n. *acidum* acid; Gr. adj. *philus* loving; M.L. adj. *acidophilum* acid-loving.

Cells are rod-shaped, $0.5\text{--}0.8 \times 1.0\text{--}1.5 \mu\text{m}$, occurring singly, mainly in pairs and rarely chains. Motile by means of single polar flagella or nonmotile. Colonies on agar media are small (1–2 mm in diameter after one week of incubation), round, smooth, convex, slightly translucent, and cream colored; if cells age, they become pink to light brown. Strictly aerobic; facultatively chemolithotroph and mixotroph, growing with elemental sulfur as an energy source. Thiosulfate, trithionate, and tetrathionate also serve as electron donor. Neither sulfite, sulfide, nor ferrous ion is used as electron donor. Polyhedral inclusion bodies (carboxysomes) are present in elemental sulfur-growing cells. Carbon sources utilized are listed in Table BXII.α.17. D-Glucose, D-fructose, and some other sugars support good growth. Growth is inhibited by the presence of 1% glucose, 0.3% yeast extract, or 1% trypticase. No growth factor is required. Ammonium salts are utilized as nitrogen source. Catalase positive. Oxidase negative. Arsenite oxidation is negative. Optimal growth occurs at 25–30°C (range: 15–35°C) and at pH 3.0–3.5 (range: pH 1.5–6.0). No growth occurs in the presence of 3% NaCl. Habitat: Originally isolated from an iron-oxidizing culture of *Thiobacillus ferrooxidans* and found in strongly acidic environments including mine drainage.

The mol% G + C of the DNA is: 62.9–63.2 (T_m).

Type strain: ATCC 27807 (DSM 700).

GenBank accession number (16S rRNA): D86511.

3. **Acidiphilium multivorum** Wakao, Nagasawa, Matsuura, Matsukura, Matsumoto, Hiraishi, Sakurai and Shiota 1995, 197^{VP} (Effective publication: Wakao, Nagasawa, Matsuura, Matsukura, Matsumoto, Hiraishi, Sakurai and Shiota 1994, 156.)

mul.ti.vo'rum. L. adj. *multus* many; L. v. *voro* eat, consume; M.L. adj. *multivorum* devouring many kinds of substances.

Cells are rod-shaped, $0.5\text{--}0.9 \times 1.5\text{--}3.8 \mu\text{m}$ under optimal growth conditions. Motile by means of single polar, subpolar, or lateral flagellum; some strains appear to be nonmotile. Colonies on agar media are circular (1–2 mm in diameter after 3 days of incubation), smooth, slightly convex, opaque, and white to cream, and turn pink to light brown after continued incubation. Strictly aerobic, chemoorganoheterotrophic bacteria. Carbon sources utilized are listed in Table BXII.α.17. D-Glucose, D-fructose, and some other sugars support good growth. Growth is not inhibited by the presence of 1% glucose, 0.3% yeast extract, or 1% trypticase. Growth factors are required and this requirement is satisfied by 0.01% yeast extract. Ammonium salts and nitrate salts are utilized as nitrogen source. Nitrate is reduced to nitrite. No denitrification. Arsenite oxidation is positive. Catalase and urease positive. Oxidase, DNase,

and amino acid carboxylases negative. Hydrogen sulfide produced. Indole not formed. Starch and gelatin are not hydrolyzed. Tween 40 and 60 hydrolyzed. Susceptible to ampicillin, chloramphenicol, penicillin, rifampin, streptomycin, and tetracycline but resistant to josamycin, kanamycin, and lincomycin. Optimal growth occurs at 27–35°C (range: 17–42°C) and at pH 3.2–4.0 (range: pH 1.9–5.6). Growth occurs in the presence of 3% NaCl. Habitat: water and sediments in acidic mine drainage streams.

The mol% G + C of the DNA is: 66.2–68.1 (HPLC).

Type strain: AIU 301, DSM 11245, JCM 8867.

GenBank accession number (16S rRNA): AB006711.

4. **Acidiphilium organovorum** Lobos, Chisolm, Bopp and Holmes 1986, 143^{VP}

or.ga.no'vor.um. N.L. n. *organum* organic, compound; L. v. *voro* eat, consume; N.L. adj. *organovorum* devouring organic compounds.

Cells are rod-shaped, $0.5\text{--}0.7 \times 1.0\text{--}1.5 \mu\text{m}$ under optimal growth conditions, occurring singly, in pairs, and in chains in some cases. Motile by means of polar or subpolar flagella. Colonies on agar media are circular (1–2 mm after 3 days of incubation), smooth, convex, slightly translucent, and white to cream, and turn pink to light brown after continued incubation. Strictly aerobic, chemoorganoheterotrophic bacteria having respiratory type of metabolism with oxygen as terminal electron acceptor. Carbon sources utilized are listed in Table BXII.α.17. D-Glucose, D-fructose, and some other sugars support good growth. Growth is not inhibited by the presence of 1% glucose, 0.3% yeast extract, or 1% trypticase. Growth factors are required, and this requirement is satisfied by 0.01% yeast extract. Ammonium and nitrate salts are utilized as nitrogen source. Catalase and urease positive. Oxidase negative. Arsenite oxidation negative. Susceptible to ampicillin, chloramphenicol, novobiocin, penicillin, rifampin, and tetracycline, but resistant to josamycin, lincomycin, and streptomycin. Optimal growth occurs at 25–30°C (range: 15–42°C) and at pH 3.0–3.5 (range: pH 2.0–6.0). Growth occurs in the presence of 3% NaCl. Habitat: Originally isolated from an iron-oxidizing culture of *Thiobacillus ferrooxidans* and found in acidic mine drainage.

The mol% G + C of the DNA is: 67.4–67.5 (HPLC).

Type strain: ATCC 43141.

GenBank accession number (16S rRNA): D30775.

5. **Acidiphilium rubrum** Wichlacz, Unz and Langworthy 1986, 200^{VP}

ru'brum. L. adj. *rubrum* red colored.

Cells are straight or slightly curved rods, $0.5\text{--}0.8 \times 1.5\text{--}3.2 \mu\text{m}$ under optimal growth conditions, occurring singly, in pairs, and in chains in some cases. Motile by means of polar or subpolar flagella. Colonies on agar media are small (1 mm in diameter after one week of incubation), round, regular, convex, slightly translucent, and red to violet. Strictly aerobic, chemoorganoheterotrophic bacteria having respiratory type of metabolism. Carbon sources utilized are listed in Table BXII.α.17. D-Glucose, D-fructose, and some other sugars support good growth. Growth is inhibited by the presence of 1% glucose, 0.3% yeast extract, or 1% trypticase. Growth factors are required and this requirement is satisfied by 0.01% yeast extract. Ammonium salts

are utilized as nitrogen source. Catalase positive. Oxidase negative. Arsenite oxidation negative. Optimal growth occurs at 25–30°C (range: 15–35°C) and at pH 3.0–3.5 (range: 2.0–5.9). No growth occurs in the presence of 3% NaCl. Habitat: water and sediments in acidic mine drainage streams.

The mol% G + C of the DNA is: 63 (T_m), 63.2–64.0 (HPLC).

Type strain: OP, ATCC 35905.

GenBank accession number (16S rRNA): D30776.

Genus III. *Acidisphaera* Hiraishi, Matsuzawa, Kanbe, and Wakao 2000b, 1544^{VP}

AKIRA HIRAISHI

A.ci.di.sphae'ra. M.L. n. *acidum* an acid; M.L. fem. n. *sphaera* sphere; M.L. fem. n. *Acidisphaera* acid (-requiring) coccoid microorganism.

Cells are cocci or coccobacilli, 0.7–0.9 × 0.9–1.6 µm. Nonmotile. Spore and capsules are not formed. Multiply by binary fission. Gram negative. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Chemoorganotrophic. **Produce bacteriochlorophyll *a* esterified with phytol and carotenoids** as photosynthetic pigments only under aerobic growth conditions in the dark. Light stimulates aerobic growth and viability. Do not grow phototrophically under anoxic conditions in the light. Liquid cultures and colonies on agar media are pink or salmon-pink. Growth occurs with a number of simple organic compounds as electron donor and carbon source. Growth on complex media containing peptone is poor. Sulfide, S⁰, thiosulfate, and Fe²⁺ do not serve as electron donors for chemolithotrophic growth. Catalase and oxidase positive. Mesophilic. **Obligately acidophilic**, growing in the pH range of 3.5–6.0 (but not at pH 3.0 or 6.5). Freshwater bacteria that do not require NaCl for optimal growth. **Ubiquinones with ten isoprene units** (Q-10) are present. Members of the class *Alphaproteobacteria*. Isolated from acidic mineral environments including surface water and sediment in acidic hot springs (at 42°C and below) and mine drainage streams.

The mol% G + C of the DNA is: 69–70.

Type species: *Acidisphaera rubrifaciens* Hiraishi, Matsuzawa, Kanbe and Wakao 2000b, 1545.

FURTHER DESCRIPTIVE INFORMATION

Cells of *A. rubrifaciens* are cocci, coccobacilli, or short rods and occur singly or in pairs (Fig. BXII.α.22). Motility is not observed at any growth stage. Thin-section electron microscopy shows that cells have typical Gram-negative membranes (Fig. BXII.α.23). Internal photosynthetic membranes are not found. Polyphosphate granules are present.

Colonies grown on agar media are 2 mm in diameter after one week of incubation, and are round, smooth, circular, convex, and salmon pink.

The photosynthetic pigments are magnesium-chelated bacteriochlorophyll (Mg-BChl) *a* esterified with phytol and the carotenoid spirilloxanthin. In addition to this carotenoid component, cells contain much larger amounts of a polar carotenoid that hardly migrates on silica-gel thin-layer chromatography with benzene–acetone (1:1, v/v) as the developing solvent. Membrane preparations from cells grown aerobically in darkness show major absorption maxima at 474–476, 502–503, 545 (shoulder), 590 (shoulder), 801, and 873–874 nm. The very low absorption peak at around 800 nm, compared to the peak at 873–874 nm, suggests that cells contain the core light-harvesting complex (LH I) together with the photosynthetic reaction center, but lack peripheral antenna complexes. The acetone–methanol extract has ab-

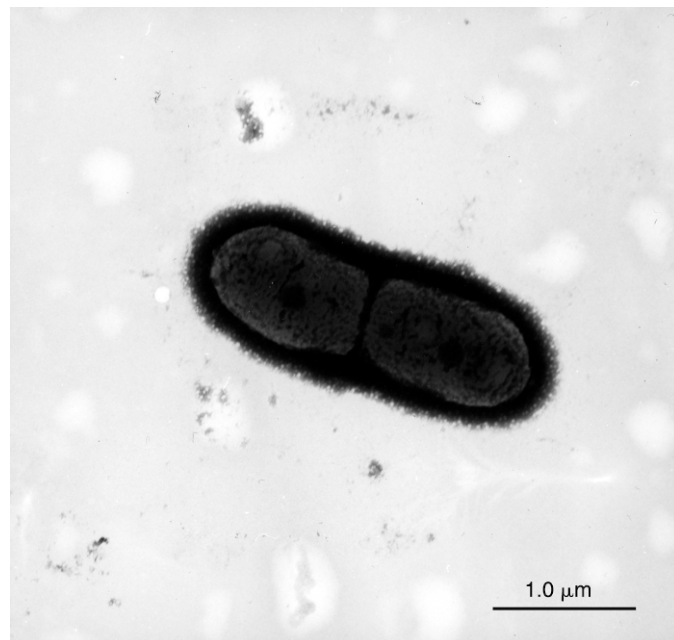


FIGURE BXII.α.22. Electron micrograph showing general cell morphology of *Acidisphaera rubrifaciens* (strain HS-AP3).

sorption maxima at 386, 466–467 (shoulder), 494–495, 525–526, and 770 nm, indicating the presence of Mg-BChl *a* but not Zn-BChl *a* as the major photopigment. However, when cells are grown in the presence of 1 mM zinc sulfate, an additional photopigment, Zn-BChl *a*, occurs in a trace amount (1–2% of the amount of Mg-BChl *a*) (Hiraishi et al., 2000b). As with many other species of aerobic BChl-containing bacteria (Shimada, 1995; Yurkov and Beatty, 1998a), aerobic and dark conditions are most favorable for BChl production by *A. rubrifaciens*. BChl production is repressed completely by continuous illumination. The BChl *a* content of *A. rubrifaciens* strains grown under optimal growth conditions in darkness ranges from 35 to 90 nmol/g dry weight of cells (Hiraishi et al., 2000b). A marked increase in BChl content is found under oligotrophic or starvation conditions (A. Hiraishi, unpublished data).

Strains of *A. rubrifaciens* are aerobic chemoorganotrophic bacteria that grow well under aerobic conditions with shaking but do not exhibit phototrophic growth under anaerobic conditions in the light. They have a doubling time of 11–13 h when grown in gluconate-containing liquid medium in darkness. Continuous incandescent illumination stimulates aerobic growth significantly, provided that the preculture is grown in darkness. The

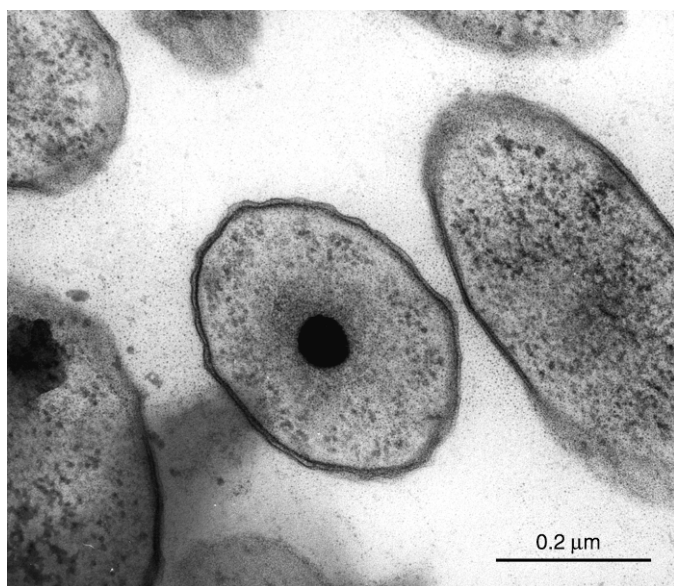


FIGURE BXII.α.23. Thin-section electron micrograph showing ultrastructure of *Acidisphaera rubrifaciens* cells (strain HS-AP3).

growth yield is 1.5–1.8-fold higher in illuminated cultures than in chemotrophic dark cultures. Illumination also enhances the viability of *A. rubrifaciens* cells under starvation conditions, as is the case in some other aerobic BChl-containing bacteria (Shiba, 1984).

The optimum pH for growth is 4.5–5.0. The temperature range for growth is 20–40°C (optimum 30–35°C). Growth is good in the absence of NaCl and is inhibited by adding 3% NaCl.

Catalase and cytochrome oxidase are produced. Hydrolysis of starch, Tween 80, or casein is negative. Nitrate is not reduced to nitrite. Utilization patterns of organic compounds as carbon and energy sources are shown in Tables BXII.α.18 and BXII.α.19. Good carbon sources are gluconate, lactate, malate, and pyru-

vate. Moderate growth is observed with xylose, galactose, glycerol, sorbitol, ethanol, and some intermediates of the tricarboxylic acid cycle. Little or no growth occurs with maltose, lactose, sucrose, lower fatty acids, citrate, benzoate, amino acids, Casamino acids, or peptone.

The major respiratory quinone, Q-10, accounts for 98–99% of the total quinone contents. The remainder detected is Q-9. Other quinone ring groups, such as menaquinones and rhodoquinones, are not found.

The natural habitats and ecological roles of *A. rubrifaciens* have not yet been fully identified. Strains of *A. rubrifaciens* have so far been isolated from acidic mineral environments including surface water and sediment in acidic hot springs (at 42°C and below) and mine drainage streams (Hiraishi et al., 2000b). In mine drainage environments, *A. rubrifaciens* frequently coexists with members of the genus *Acidiphilium*, another group of acidophilic aerobic BChl-containing bacteria (A. Hiraishi, unpublished observations). In view of its characteristic lifestyle as an acidophilic BChl-producing bacterium, *A. rubrifaciens* appears to have an ecological niche similar to that of *Acidiphilium* species, although the former organism differs from the latter in exhibiting weaker acidophily and lacking Zn-BChl as the main photosynthetic pigment. In sunlight-exposed environments, *A. rubrifaciens* can be viable and survive for a long time without any carbon/energy source. Synthesis and expression of the photosynthetic apparatus in *A. rubrifaciens* may be regarded as a result of its adaptation to oligotrophic and mineral environments like hot springs and mine drainage streams.

ENRICHMENT AND ISOLATION PROCEDURES

A suitable medium for growth and purification of *Acidisphaera* contains (per liter distilled water): (NH₄)₂SO₄, 1.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.05 g; and trace element solution SL8 (Biebl and Pfennig, 1978), 1.0 ml. To this medium are added (per liter): gluconate, 2.0–3.0 g, and yeast extract (Difco), 1.0 g. The organic supplements are autoclaved separately and combined with the basal medium before use. The final pH of the medium should be 4.0–4.5. For solid media, separately

TABLE BXII.α.18. Differential characteristics of *Acidisphaera* and phenotypically and phylogenetically related genera of BChl-containing bacteria^a

Characteristic	<i>Acidisphaera</i>	<i>Acidiphilium</i> ^b	<i>Craurococcus</i> ^c	<i>Paracraurococcus</i> ^c	<i>Roseococcus</i> ^d	<i>Rhodopila</i> ^e
Cell shape	Cocci, short rods	Rods	Cocci	Cocci	Cocci	Cocci
Cell diameter (μm)	0.7–0.9	0.5–0.7	0.8–2.0	0.8–1.5	0.9–1.3	1.6–1.8
Motility	–	+	–	–	+	+
Color of colonies	Salmon-pink	Pink, red	Pink	Red	Pink	Purple-red
Zn-BChl as main pigment	–	+	–	–	–	–
Near IR peak for BChl a (nm)	874	864	872	856	859	865
Anaerobic phototrophy	–	–	–	–	–	+
Optimum pH for growth	4.5–5.0	3.0–3.5	7.5	6.6–6.8	7–8	4.8–5.0
Growth at pH 7	–	–	+	+	+	–
<i>Carbon source:</i>						
Acetate	–	–	–	–	+	–
Succinate	+	D	–	–	+	+
Major quinones	Q-10	Q-10	Q-10	Q-10	Q-10	Q-9(10), MK-9(10), RQ-9(10)
Mol% DNA G + C content	69.1–69.8	62.9–68.3	70.5	70.3–71.0	70.4	66.3
Habitat	Acidic springs and mine water	Acidic mine drainage	Soil	Soil	Cyanobacterial mats in sulfur spring	Acidic sulfur spring

^aFor symbols see standard definitions; Q-10, ubiquinone-10; MK-9, menaquinone-9; RQ-9, rhodoquinone-9. Q-9(10), MK-9(10), and RQ-9(10) mean the presence of those quinones with both 9 and 10 isoprene units in the side chain.

^bInformation from Hiraishi et al. (1998).

^cInformation from Saitoh et al. (1998).

^dInformation from Yurkov and Gorlenko (1992) and Yurkov et al. (1994c).

^eInformation from Pfennig (1974).

autoclaved 1.5% agar or 0.6% gellan gum can be used. Enrichment and isolation of *A. rubrifaciens* from the environment are possible using the above-noted medium, but an alternative use of both gluconate (1.0 g/l) and D-xylose (1.0 g/l) as the carbon source may be more effective in some cases. Cultures are incubated aerobically at 30°C in darkness. Although other aerobic acidophilic bacteria and acid tolerant bacteria are frequently recovered in or on isolation media, enriched cultures or colonies of *A. rubrifaciens* are distinguishable by their pink or salmon-pink color.

MAINTENANCE PROCEDURES

Cultures are preserved in liquid nitrogen or by lyophilization. Preservation in a mechanical freezer at -80°C is also possible.

DIFFERENTIATION OF THE GENUS *ACIDISPHAERA* FROM OTHER GENERA

The genus *Acidisphaera* is differentiated from other genera of aerobic BChl-containing bacteria by a combination of morphological, physiological, and chemotaxonomic characteristics as indicated in Table BXII.α.18.

TAXONOMIC COMMENTS

The description of the genus *Acidisphaera* is based on only one species, *A. rubrifaciens* (Hiraishi et al., 2000b). The 16S rDNA sequences of *A. rubrifaciens* strains place them in the major acidophilic cluster of the class *Alphaproteobacteria*, with members of the genera *Acidiphilium*, *Rhodopila*, *Acetobacter*, and *Gluconobacter* as their phylogenetic relatives. The nearest phylogenetic neighbor is the anaerobic phototrophic bacterium *Rhodopila globiformis* (95% similarity).

Before the description of *A. rubrifaciens*, acidophilic aerobic BChl-containing bacteria had been limited to members of the genus *Acidiphilium*, which contain Zn-BChl *a* rather than Mg-BChl *a* as the major photopigment (Wakao et al., 1996; Hiraishi et al., 1998). Thus, *A. rubrifaciens* is the first description of acidophilic aerobic bacteria containing Mg-BChl *a* as the major photopigment. *A. rubrifaciens* is similar to the moderately acidophilic phototrophic bacterium *Rhodopila globiformis* (Pfennig, 1974) in morphology, physiology, and natural habitats, but *A. rubrifaciens*

TABLE BXII.α.19. Physiological and biochemical characteristics of *Acidisphaera rubrifaciens*^a

Characteristic	Result/reaction
Optimal temperature (°C) for growth	30–35
Optimal pH for growth (range)	4.5–5.0 (3.5–6.0)
Growth in the presence of:	
0–1% NaCl	+
3% NaCl	–
Vitamin requirement for growth	+
Nitrate reduction to nitrite	–
Catalase	+
Cytochrome oxidase	+
Hydrolysis of starch, casein, or Tween 80	–
Utilization of carbon/energy sources:	
Sugars and sugar alcohols:	
L-Arabinose, D-fructose, and mannitol	d
D-Galactose, D-glucose, glycerol, sorbitol, and D-xylose	+
Inositol, lactose, maltose, D-mannose, and sucrose	–
Alcohols:	
Ethanol	+
Methanol, propanol, and butanol	–
Organic acids:	
Benzoate, citrate, and lower fatty acids	–
Fumarate, gluconate, lactate, malate, pyruvate, and succinate	+
Amino acids and others:	
Alanine, asparagine, aspartate, glutamate, leucine, Casamino Acids, and peptone	–
Yeast extract	+

^aSymbols: see standard definitions.

differs clearly from the latter by failing to grow anaerobically in the light. The level of 16S rDNA sequence similarity between *A. rubrifaciens* and *R. globiformis* is low enough to warrant different generic allocations.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ACIDISPHAERA*

The genus *Acidisphaera* is monotypic, with *A. rubrifaciens* as the type species. General characteristics of *A. rubrifaciens* in terms of physiology and biochemistry are shown in Table BXII.α.19.

List of species of the genus *Acidisphaera*

1. ***Acidisphaera rubrifaciens*** Hiraishi, Matsuzawa, Kanbe and Wakao 2000b, 1545^{VP}
rub.ri.fac'i.ens. L. adj. *ruber* red; L. v. *facio* make; M.L. part. adj. *rubrifaciens* red-producing.

The characteristics are as described for the genus and as listed in Tables BXII.α.18 and BXII.α.19, with the following additional features. Cells are 0.7–0.9 × 0.9–1.6 μm. No intracytoplasmic membrane systems occur. Polyphosphate granules are present. Cell membranes have absorption maxima at 474–476, 502–503, 545, 590, 801, and 873–874 nm. Acetone-methanol extracts from cells have absorp-

tion maxima at 386, 466–467, 494–495, 525–526, and 770 nm. Sprilloxanthin and a polar pigment are the major carotenoids. No hydrolysis of starch, casein and Tween 80 occurs. Gluconate, lactate, malate, and pyruvate are good carbon sources. Other usable carbon sources are galactose, glucose, D-xylose, glycerol, mannitol, sorbitol, fumarate, malate, succinate, and yeast extract. The habitats are strongly acidic mineral environments, including acidic hot springs (at 42°C and below) and pyritic mine drainage.

The mol % G + C of the DNA is: 69.1–69.8 (HPLC).

Type strain: HS-AP3, JCM 10600.

GenBank accession number (16S rRNA): D86512.

Genus IV. *Acidocella* Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1996, 362^{VP} (Effective publication: Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1995b, 90)

AKIRA HIRAISHI

A.ci.do.cel'la. M.L. n. *acidum* an acid; L. n. *cella* a cell; M.L. n. *Acidocella* acid (-requiring) cell.

Straight or slightly curved rods and coccobacilli with rounded or tapered ends, $0.5\text{--}0.8 \times 1.0\text{--}2.0\ \mu\text{m}$. Motile by means of polar or lateral flagella; some strains are nonmotile. Nonsporeforming and nonencapsulated. Gram negative. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Cell suspensions and colonies are white, cream, or light brown. Photosynthetic pigments and carotenoids are absent. Mesophilic. **Obligately acidophilic, growing in the pH range of 3.0–6.0**; some strains grow at pH 2.5. No growth occurs at pH ≥ 6.1 . **Chemoorganotrophs**, growing with simple organic compounds as carbon and energy sources. **Growth occurs in the presence of either 0.25 mM acetate, 2 mM lactate, or 4 mM succinate**. Catalase positive. **Oxidase negative or weakly positive**. **A straight-chain monounsaturated C_{18:1} acid is the major component of cellular fatty acids**. Ubiquinones with ten isoprene (Q-10) units are present. **16S rRNA structures and signatures conform to species of the *Alphaproteobacteria***. Inhabit strongly acidic mineral environments.

The mol% G + C of the DNA is: 58–65.

Type species: *Acidocella facilis* (Wichlacz, Unz, and Langworthy 1986) Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1996, 362 (Effective publication: Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1995b, 90) (*Acidiphilium facilis* Wichlacz, Unz, and Langworthy 1986, 200.)

FURTHER DESCRIPTIVE INFORMATION

Acidocella cells are straight or slightly curved rods with rounded or tapered ends, occurring singly or in pairs (Fig. BXII.α.24). In

some cases, they are observed in chains and small flocs. Cells vary from 0.5 to 0.8 μm in width and from 1.0 to 2.0 μm in length under optimal growth conditions. Strains of each species are usually motile, but nonmotile strains are occasionally found. Electron microscopy of negatively stained cells shows that motile cells have polar or lateral flagella (Fig. BXII.α.25). The flagellar arrangement is different from strain to strain; in some cases, cells with polar flagella and with lateral flagella coexist in a single strain.

Colonies on agar media are circular (3–5 mm in diameter after 3 d of incubation), smooth, convex, slightly translucent, and white, cream, or light brown in color. Neither bacteriochlorophylls nor carotenoid pigments are present.

Acidocella species are nutritionally more versatile and exhibit more rapid and luxuriant growth than the phylogenetically and physiologically related *Acidiphilium* species. Most *Acidocella* strains exhibit a doubling time of 2–3 h under optimal growth conditions. The following sugars and sugar alcohols are good substrates supporting growth of all species: L-arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-fructose, sucrose, glycerol, arabitol, and mannitol. The following compounds are not used as carbon sources: L-rhamnose, maltose, cellobiose, methanol, formate, acetate, propionate, butyrate, valerate, caproate, lactate, and benzoate. As is the case with *Acidiphilium*, acidophilic growth of *Acidocella* strains is inhibited in the presence of high concentrations of organic acids such as acetate, lactate, and succinate when grown in glucose-mineral medium. However, *Acidocella* species are more tolerant to such organic acids at low concentrations.

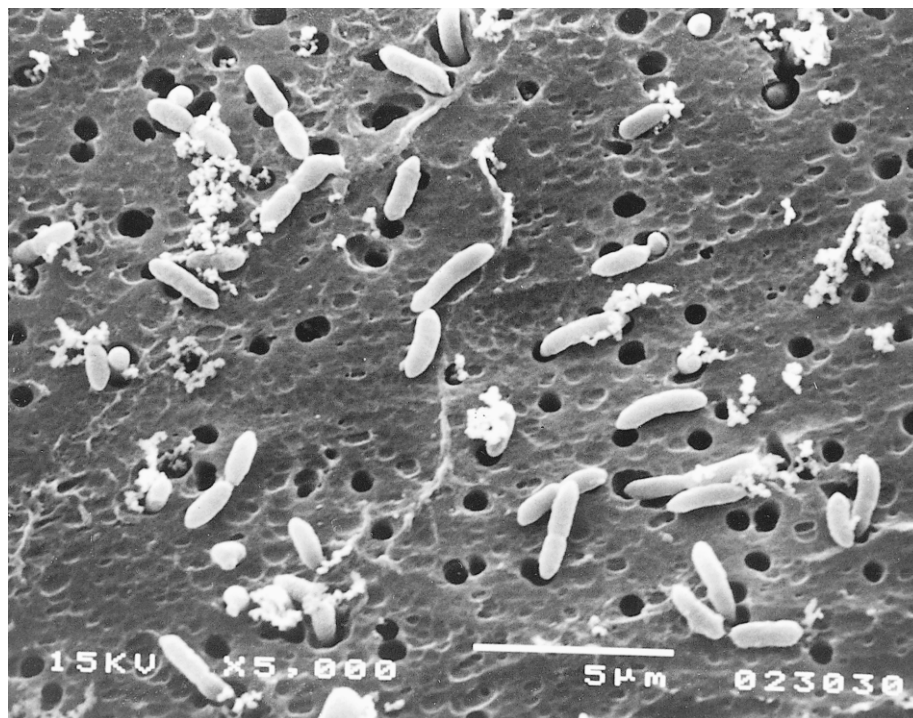


FIGURE BXII.α.24. Scanning electron micrograph showing general cell morphology of *Acidocella facilis* (ATCC 35904^T.)

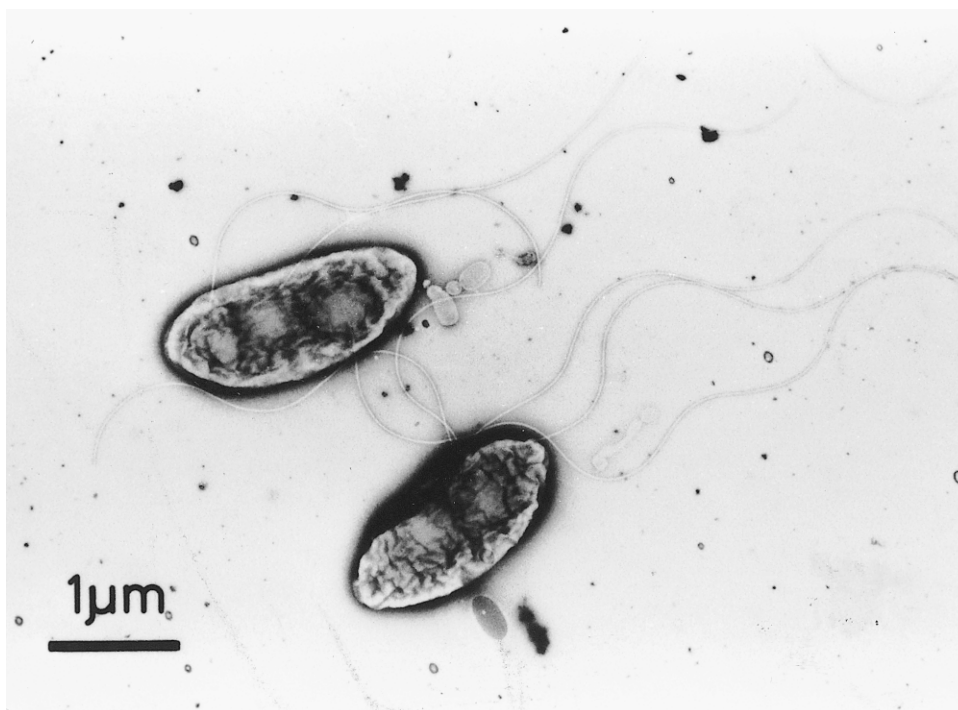


FIGURE BXII.α.25. Electron micrograph of negatively stained cells of *Acidocella facilis* (ATCC 35904^T) having lateral flagella. (Reprinted with permission from K. Inagaki, Nippon Nogekagaku Kaishi 71: 1–8, 1997, ©Japan Society for Bioscience, Biotechnology, and Agrochemistry.)

They are capable of growing in the presence of 0.25 mM acetate, 2 mM lactate, 4 mM succinate, or 0.3% yeast extract, each of which has an inhibitory effect on the growth of *Acidiphilium*.

None of the *Acidocella* species grows chemolithotrophically with S^0 or thiosulfate as the energy source. Neither Fe^{2+} nor other metal ions serve as the electron donor for growth. However, the addition of ferrous or aluminum ions stimulates heterotrophic growth significantly. All species are capable of growth in the presence of 100 mM Fe^{2+} and 100 mM Al^{3+} . Growth yields are much higher in the presence of 100 mM Al^{3+} than in the absence of Al^{3+} , and this growth-promoting effect is more pronounced with aluminum sulfate than with aluminum chloride or aluminum phosphate (A. Hiraishi, unpublished). Some strains also exhibit extremely high resistance to heavy metals such as cadmium, copper, nickel, and zinc; however, these metals extend the lag period and generation time for growth. The heavy-metal resistance of *Acidocella* strains may be plasmid-dependent. When *Acidiphilium multivorum* and *Escherichia coli* were subjected to transformation with a plasmid prepared from an *Acidocella* strain, they became more highly resistant to cadmium and zinc (Ghosh et al., 1997).

Acidocella strains, as well as *Acidiphilium* strains, contain high amounts of phosphorus-free lipoamino acids in their cell membranes. Ornithine amide lipids, $C_{18:1}$ fatty acid esters of α -*N*-3-hydroxystearylornithine and α -*N*-3-hydroxystearylornithyltaurine, are the major polar aminolipids (Kishimoto et al., 1993a). In addition to these lipids, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine are present as the major phospholipids. Bisphosphatidylglycerol is absent. Straight-chain monounsaturated $C_{18:1}$ acid is the major component of whole-

cell fatty acids, constituting 57–66% of the total content (Kishimoto et al., 1993b). A considerable amount of cyclopropane $C_{19:0}$ acid (6–21%) is also present. 2-Hydroxymyristic acid is found in these strains. Ubiquinone-10 is the sole respiratory quinone.

The genetic aspects of *Acidocella facilis* have been studied to some degree. The *recA* gene of *A. facilis* has been cloned and sequenced (Inagaki et al., 1993). In the deduced amino acid sequence of the *recA* protein, *A. facilis* showed a similarity level of 72% to *Thiobacillus ferrooxidans*, 62% to *Escherichia coli*, and 53% to *Bacillus subtilis*.

Like *Acidiphilium* strains, *Acidocella* strains inhabit strongly acidic mineral environments. These organisms have so far been isolated from water and sediment taken from acidic mine drainage streams (Wichlacz et al., 1986; Kishimoto et al., 1993b) and acidic soil from a copper mine (Banerjee et al., 1996). In these environments, *Acidocella* strains frequently coexist with members of the genera *Acidiphilium* and *Acidithiobacillus*. *Acidocella* strains also inhabit acidic hot springs and acidic farm soil, and are the most abundant heterotrophic bacteria isolated from sulfuric acidic tropical soil (I. Nioh, personal communication). Moreover, mud and sewage at slightly acidic pH are possible sources of these organisms (Kishimoto and Tano, 1987; Kishimoto et al., 1993b).

ENRICHMENT AND ISOLATION PROCEDURES

Growth media for *Acidocella* are similar to those for *Acidiphilium*. A suitable medium consists of (per liter of distilled water): $(NH_4)_2SO_4$, 2.0 g; KH_2PO_4 , 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; and trace element solution SL8 of Biebl and Pfennig (1978) (pH 3.0), 1.0 ml. Glucose (3.0 g/l) and yeast

extract (Difco, 0.5 g/l) are added to the medium from autoclaved stock solutions to give final concentrations of 3.0 g/l and 0.5 g/l, respectively. When used as a solidified medium, the medium also contains 1.5% agar (separately autoclaved). Alternatively, 0.6% gellan gum can be used for this purpose (Kishimoto and Tano, 1987). For selective isolation and enrichment of *Acidocella* from the environment, the medium is supplemented with 0.2 mM acetate to inhibit co-growth of *Acidiphilium* and is adjusted to pH 2.5–3.0 to minimize growth of fungi and facultatively acidophilic bacteria.

MAINTENANCE PROCEDURES

Cultures can be preserved in liquid nitrogen or by lyophilization. Preservation in a mechanical freezer at -80°C is also satisfactory.

DIFFERENTIATION OF THE GENUS *ACIDOCELLA* FROM OTHER GENERA

The genus *Acidocella* is separated from other genera of chemorganotrophic proteobacteria by its phylogenetic position and its outstanding physiological and chemotaxonomic characteristics. The differential characteristics of the genera *Acidocella* and *Acidiphilium* are indicated in Table BXII.α.20.

TAXONOMIC COMMENTS

Phylogenetic analyses based on 16S rDNA sequence information have shown that *Acidocella* species form a major cluster in the *Alphaproteobacteria*, together with the acidophilic, chemotrophic genera *Acidiphilium*, *Acetobacter*, and *Gluconobacter*, and the anaerobic phototrophic acidophile *Rhodospira* (Sievers et al., 1994a; Kishimoto et al., 1995b). The genus *Acidiphilium* is the nearest phylogenetic neighbor. The levels of 16S rDNA sequence similarity between members of the genera *Acidocella* and *Acidiphilium* are 92–94%.

The two established species of the genus *Acidocella*, *A. facilis* and *A. aminolytica*, were originally classified in the genus *Acidiphilium* as *Acidiphilium facilis* and *Acidiphilium aminolytica*, respectively. However, these two species were found to differ from other *Acidiphilium* species in the lack of the ability to produce photosynthetic pigments (Wakao et al., 1993; Kishimoto et al., 1995a)

TABLE BXII.α.20. Differential characteristics of the genera *Acidocella* and *Acidiphilium*^a

Characteristic	<i>Acidocella</i>	<i>Acidiphilium</i>
Cell width, μm	0.5–0.8	0.3–0.8
Pink or yellow pigment	—	D
Photosynthetic pigments	—	+
Growth inhibition by:		
Acetate, 0.25 mM	—	+
Lactate, 2 mM	—	+
Succinate, 4 mM	—	+
Bisphosphatidylglycerol	—	+
2-Hydroxy fatty acid	C _{14:0}	—
Mol% G + C of DNA	58–65	63–68

^aFor symbols see standard definitions.

and in some other phenotypic properties (Kishimoto et al., 1995b). Also, phylogenetic analyses based on 16S rDNA sequences revealed that although *A. facilis* and *A. aminolytica* form a monophyletic cluster with other *Acidiphilium* species in the *Alphaproteobacteria*, there was a significant genetic distance at the generic level between the former two species and the latter (Kishimoto et al., 1995b). These findings led to the conclusion that the non-BChl producing species (i.e., *A. facilis* and *A. aminolytica*) should be removed from the genus *Acidiphilium*.

However, the name *Acidocella* is an orthographic error. The first constituent of the name (Acidi-, from *acidum*, acid) is derived from Latin and therefore the connecting vowel to the second constituent (-cella cell) must be -i-. A correction of the name *Acidocella* to *Acidicella* is recommended so as to be in accord with Rule 57c, Note 1 of the International Code of Nomenclature of Bacteria (Lapage et al., 1992). Likewise, the original spelling of the specific epithet, *A. aminolytica*, should be changed to *amini-lytica*.

The levels of 16S rDNA sequence similarity and DNA–DNA hybridization between *A. facilis* and *A. aminolytica* are 98% and 10–15%, respectively (Kishimoto et al., 1993b, 1995b).

ACKNOWLEDGMENTS

The author is indebted to K. Inagaki for providing the electron micrograph and H.G. Trüper for his valuable advice on bacterial nomenclature.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ACIDOCELLA*

The differential characteristics of the species of *Acidocella* are indicated in Table BXII.α.21.

List of species of the genus *Acidocella*

- 1. *Acidocella facilis*** (Wichlacz, Unz, and Langworthy 1986) Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1996, 362^{VP} (Effective publication: Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1995b, 90) (*Acidiphilium facilis* Wichlacz, Unz, and Langworthy 1986, 200.)
fa'ci.lis. L. adj. *facilis* ready, quick, with respect to growth.

The characteristics are as described for the genus and indicated in Tables BXII.α.21 and BXII.α.22. Habitat: acidic mine drainage and acidic soil.

The mol% G + C of the DNA is: 64.0–64.4 (HPLC).

Type strain: Strain PW2, ATCC 35904.

GenBank accession number (16S rRNA): D30774.

2. **Acidocella aminolytica** (Kishimoto, Kosako and Tano 1993b) Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1996, 362^{VP} (Effective publication: Kishimoto, Kosako, Wakao, Tano, and Hiraishi 1995b, 90) (*Acidiphilium aminolytica* Kishimoto, Kosako, and Tano 1993b, 135.)
a.mi.no.ly'ti.ca. M.L. n. *aminum* amine; Gr. adj. *lytica* dissolving; M.L. adj. *aminolytica* amine dissolving.

The characteristics are as described for the genus and indicated in Tables BXII.α.21 and BXII.α.22. Habitat: acidic mine drainage and mud.

The mol% G + C of the DNA is: 58.7–59.2 (HPLC).

Type strain: Strain 101, ATCC 51361, DSM 11237, JCM 8796.

GenBank accession number (16S rRNA): D30771.

TABLE BXII.α.21. Differential characteristics of the species of the genus *Acidocella*^a

Characteristic	<i>A. facilis</i>	<i>A. aminolytica</i>
Growth with 3.5% NaCl	+	–
Hydrolysis of hippurate	–	+
Oxidase	d	–
<i>Carbon source utilization:</i>		
Lactose, ethanol	+	–
Sorbitol, inositol, alanine, lysine, spermine	–	+
Glycerol	+	d
Creatine	–	d
Mol% G + C of DNA	58.7–59.2	64.0–64.4

^aFor symbols see standard definitions.

TABLE BXII.α.22. Other characteristics of the species of the genus *Acidocella*^a

Characteristic	<i>A. facilis</i>	<i>A. aminolytica</i>
Cell width, μm	0.6–0.8	0.5–0.8
Motility by flagella	+	+
Pigmentation	–	–
pH range for growth	3.0–6.0	3.0–6.0
Growth at 37°C	+	+
Chemolithotrophic growth with Fe ²⁺ or S ⁰	–	–
Hydrolysis of esculin	–	–
Oxidase	d	–
Catalase	+	+
<i>Carbon source utilization:</i>		
L-Arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-fructose, arabinol, mannitol, succinate, diaminobutane, DL-4-aminobutyrate, DL-5-aminovalerate, arginine	+	+
L-Rhamnose, maltose, cellobiose, starch, methanol, formate, acetate, lactate, glutamate, glycine	–	–
Pyruvate	d	nd
Citrate, <i>cis</i> -aconitate, α-ketoglutarate, fumarate, malate	+	nd
Gluconate	nd	+
Casamino acids, peptone, yeast extract	+	+
Major fatty acid	C _{18:1}	C _{18:1}
Major quinone	Q-10	Q-10

^aFor symbols see standard definitions; nd, not determined; Q-10, ubiquinone-10.

Genus V. *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989a, 54^{VP}

MARTIN SIEVERS AND JEAN SWINGS

A.ci.do.mo' nas. Gr. adj. *acid* acid; Gr. n. *monas* unit, monad; M.L. fem. n. *Acidomonas* acidophilic monad.

Cells are Gram negative, nonsporeforming, nonmotile, rod shaped, 0.8–1.0 × 1.5–3.0 μm. Cells occur singly, rarely in pairs. Colonies were white to yellow on PYM medium. Strictly aerobic. Facultative methylotroph. Metabolism is respiratory, never fermentative. Optimal temperature for growth is 30°C. Optimal pH for growth is pH 4.0–4.5. Catalase positive. Nitrate is not reduced to nitrite. Oxidizes ethanol to acetic acid. Overoxidizes acetic acid to CO₂ and H₂O. *Acidomonas* contains C_{18:1} straight-chain unsaturated fatty acid as well as C_{16:0 3OH} and C_{16:0 2OH} as major components of cellular and hydroxy fatty acids, as do the other genera of the family *Acetobacteraceae* (Yamada et al., 1981; Urakami and Komagata, 1987a; Urakami et al., 1989a). The ubiquinone system is Q-10, along with ubiquinone Q-9 and Q-11 as minor components.

The mol% G + C of the DNA is: 62.

Type species: ***Acidomonas methanolica*** (Uhlig, Karbaum and Steudel 1986) Urakami, Tamaoka, Suzuki and Komagata 1989a, 54 (*Acetobacter methanolicus* Uhlig, Karbaum and Steudel 1986, 321.)

MAINTENANCE PROCEDURES

For cultivation and maintenance of *Acidomonas* a peptone–yeast extract–malt extract agar (PYM medium) is used. The medium is composed of 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1.0% glucose, and 2.0% agar; adjusted to pH 4.5 with 1 N HCl (Urakami et al., 1989a).

Cultures were incubated at 30°C. In addition, the medium 569 is used for cultivation of *Acidomonas methanolica*. It is composed of: KNO₃, 1.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.02 g; Na₂HPO₄, 0.23 g; NaH₂PO₄, 0.07 g; FeSO₄·7H₂O, 1.0 mg; CuSO₄·5H₂O, 5.0 mg; H₃BO₃, 10.0 mg; MnSO₄·5H₂O, 10.0 mg; ZnSO₄·7H₂O, 70 mg; MoO₃, 10 mg; agar, 12 g; distilled H₂O, 1000 ml; methanol, 10 ml. Sterile-filtrated methanol is added after sterilization of the basal medium. The pH of the medium is 6.8, growth is aerobically at 30°C.

DIFFERENTIATION OF THE GENUS *ACIDOMONAS* FROM OTHER GENERA

Acidomonas can be differentiated from *Acetobacter*, *Gluconacetobacter*, *Asaia*, and *Gluconobacter* by growth on methanol and utilization of methanol.

TAXONOMIC COMMENTS

Acetobacter methanolicus was described by Uhlig et al. (1986) as a new *Acetobacter* species and classified as *Acidomonas methanolica* by Urakami et al. (1989a). It was reclassified in the genus *Acetobacter* by Sievers et al. (1994b) based on the 16S rDNA sequence of the type strain. Due to the current classification of acetic acid bacteria (Table BXII.α.9 in *Acetobacteraceae*), the name *Acidomonas methanolica* is used.

List of species of the genus *Acidomonas*

1. ***Acidomonas methanolica*** (Uhlrig, Karbaum and Steudel 1986) Urakami, Tamaoka, Suzuki and Komagata 1989a, 54^{VP} (*Acetobacter methanolicus* Uhlrig, Karbaum and Steudel 1986, 321.)

me.tha.no'li.cus. L. n. *methanolum* methanol; L. adj. *methanolicus* methanolic, using methanol as a sole carbon source.

Ellipsoidal to rod-shaped cells 0.6–0.8 × 1.0–1.8 µm, occurring singly, in pairs, or rarely in short chains. Cells are usually motile by peritrichous flagellation. Oxidase negative (but under methylotrophic conditions oxidase positive). *A. methanolica* is a facultatively methylotrophic bacterium and utilizes methanol as the sole source of carbon and energy. Methanol is oxidized to formate and fixed by the ribulose monophosphate cycle for biosynthetic purposes (Steudel et al., 1980; Babel, 1984). *A. methanolica* has two independent respiratory chains for methanol and ethanol (Matsushita et al., 1992c). Two different forms of methanol dehydrogenase have been purified from *A. methanolica* containing PQQ as cofactor (Matsushita et al., 1993). Glucose is oxidized to gluconate, but 2-, 5-ketogluconic acid, and 2,5-diketogluconic acid are not produced. No production of dihydroxyacetone from glycerol; γ-pyrones and brown pigments are not formed. *A. methanolica* utilizes methanol, ethanol, acetic acid, D-glucose, glycerol, and pectin as sole carbon sources. Growth did not occur on D-fructose, D-sorbitol, D,L-lactate, sucrose, and lactose. Growth occurs between pH 2.0 and 5.5. Weak growth occurs on D-mannitol and D-mannose. Acid is produced oxidatively from glucose, ethanol, and methanol, but not from D-mannitol, D-xylose, L-arabinose, D-mannose, D-fructose, D-galactose, D-sorbitol, sucrose, and lactose. A medium composed of 0.3% peptone, 0.5% yeast extract, 0.1% D-glucose, and 1.5% methanol (pH adjusted to 5.5) is used for cultivation of the strains.

The mol% G + C of the DNA is: 62 (T_m).
Type strain: TK 0705, ATCC 43581, DSM 5432, IMET 10945; MB 58.
GenBank accession number (16S rRNA): D30770, X77468.

Genus VI. *Asaia* Yamada, Katsura, Kawasaki, Widyastuti, Saono, Seki, Uchimura and Komagata 2000, 828^{VP}

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A.sa'i.a. M.L. fem. n. *Asaia* derived from Toshinobu Asai, a Japanese bacteriologist who contributed to the systematics of acetic acid bacteria.

Cells are aerobic and rod shaped. Peritrichously flagellated when motile. Does not produce acid from ethanol (with the exception of one strain oxidizing ethanol to acetic acid weakly). Growth is inhibited by 0.35% (v/v) acetic acid. *Asaia* weakly oxidizes acetate and lactate to carbon dioxide and water. No growth on methanol. Growth occurred on mannitol. Produces 2-keto-D-gluconate and

5-keto-D-gluconate but not 2,5-diketo-D-gluconate from D-glucose. Acids are produced from D-glucose, D-fructose, L-sorbose, D-mannitol, and D-sorbitol. The major quinone type is Q-10.

The mol% G + C of the DNA is: 59–61.

Type species: ***Asaia bogorensis*** Yamada, Katsura, Kawasaki, Widyastuti, Saono, Seki, Uchimura and Komagata 2000, 828.

List of species of the genus *Asaia*

1. ***Asaia bogorensis*** Yamada, Katsura, Kawasaki, Widyastuti, Saono, Seki, Uchimura and Komagata 2000, 828^{VP}
bo.go.r'en.sis. M.L. adj. *bogorensis* derived from Bogor, Java, Indonesia, where most of the strains were isolated.

Cells are 0.4–1.0 × 0.8–2.0 µm in size. Colonies are pink-yellowish white, shiny, smooth, and raised with an entire margin on AG agar plates (Yamada et al., 2000). AG medium is composed of 0.1% D-glucose, 1.5% glycerol, 0.5% peptone, 0.5% yeast extract, 0.2% malt extract, 0.7% CaCO₃ and 1.5% agar (Yamada et al., 2000). *A. bogorensis* produces acid from D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-xylose, ribitol, *meso*-erythritol, glycerol, and melibiose. No acid production from lactose. *A. bogorensis* produces acid from dulcitol and assimilates dulcitol for growth. Acid production from D-mannitol and D-sorbitol is strain dependent. 16S rRNA sequence similarity value of *A. bogorensis* IFO 16594 with the type strain of *Acidomonas methanolica* is 96.2%.

The mol% G + C of the DNA is: 59–61 (T_m).

Type strain: IFO 16594, JCM 10569, NRIC 0311.

GenBank accession number (16S rRNA): AB025928.

2. ***Asaia siamensis*** Katsura, Kawasaki, Potacharoen, Saono, Seki, Yamada, Uchimura and Komagata 2001, 562^{VP}
si.am'en.sis. M.L. adj. *siamensis* of Siam, old name of Thailand.

Asaia siamensis was isolated from tropical flowers collected in Thailand and Indonesia. Isolates were cultivated for enrichment on sorbitol and dulcitol medium at pH 3.5 (Yamada et al., 2000). Cells are 0.6–1.0 × 1.0–4.5 µm. Colonies are pink, shiny, smooth, and raised with an entire margin on AG agar plates (Katsura et al., 2001). *A. siamensis* produces acid from different sugars (D-glucose, D-mannose, D-fructose, L-sorbose, D-xylose, D-mannitol, D-sorbitol, glycerol, sucrose) but not from lactose and dulcitol. Strains assimilate dulcitol for growth. Acid production from dulcitol is used for differentiation of *A. bogorensis* from *A. siamensis*. 16S rRNA sequence similarity value of *A. siamensis* IFO 16457 with the type strain of *A. bogorensis* is 99.8% by DNA relatedness of 20% and 33%, respectively.

The mol% G + C of the DNA is: 59–60 (T_m).

Type strain: IFO 16457, JCM 10715, NRIC 0323.

GenBank accession number (16S rRNA): AB035416.

Genus VII. Craurococcus Saitoh, Suzuki and Nishimura 1998, 1044^{VP}

CHRISTOPHER RATHGEBER AND VLADIMIR V. YURKOV

Cra.u.ro.coc'cus. Gr. adj. *crauros* fragile; Gr. n. *coccus* berry; M.L. n. *Craurococcus* fragile coccus.

Cells are coccoid, 0.8–2.0 μm in diameter, nonmotile, Gram negative, and divide by binary fission. Form pink irregular colonies when grown on agar media. Contain bacteriochlorophyll (Bchl) *a*, spirilloxanthin and carotenoid acids. No growth occurs anaerobically in the light. Grow heterotrophically under aerobic conditions. Nitrate is reduced to nitrite, dissimilatory denitrification not observed.

The mol% G + C of the DNA is: 70.5.

Type species: *Craurococcus roseus* Saitoh, Suzuki and Nishimura 1998, 1045.

FURTHER DESCRIPTIVE INFORMATION

C. roseus is the only species presently described within the genus *Craurococcus*. Phylogenetically *Craurococcus* forms a cluster with *Paracraurococcus*, *Roseococcus*, *Rhodopila*, and *Acidiphilium* within the *Alphaproteobacteria* (Saitoh et al., 1998).

C. roseus forms irregular light yellowish-red to pink colonies, less than 1 mm in diameter, on agar medium. Cells of *C. roseus* grown in NPGC medium (Saitoh and Nishimura, 1996) adjusted

to pH 7.6 were coccoid. The cell size ranges from 0.8 to 2.0 μm in diameter. Cells are nonmotile, occur singly or in pairs, rarely in tetrads. Spore production has not been observed. Cells accumulate poly- β -hydroxybutyrate (PHB) granules when grown under usual culture conditions. Suspension in aqueous solution free of divalent cations causes cells to lyse (Fig. BXII.α.26). Divalent cations such as Ca^{2+} and Mg^{2+} seem to be necessary to maintain cell structure and prevent cell lysis. In liquid culture, divalent cations (0.1–1.0 g/l of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) are required for growth, and optimal growth occurs in BS-XY (Saitoh and Nishimura, 1996) media supplemented with 0.5 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Supplemental cations are not required for growth on agar media.

In vivo absorption spectra of *C. roseus* show absorption peaks at 800 and 872 nm, indicating the presence of Bchl *a* organized into reaction center and light-harvesting complexes, respectively. Crude pigment extracts in methanol give absorption spectra peaks at 362, 492, and 607 nm due to the presence of carotenoid pigments, as well as a 770 nm peak due to Bchl *a*. Bchl *a* content in cells is estimated to be 0.13 nmol/mg dry cell weight when

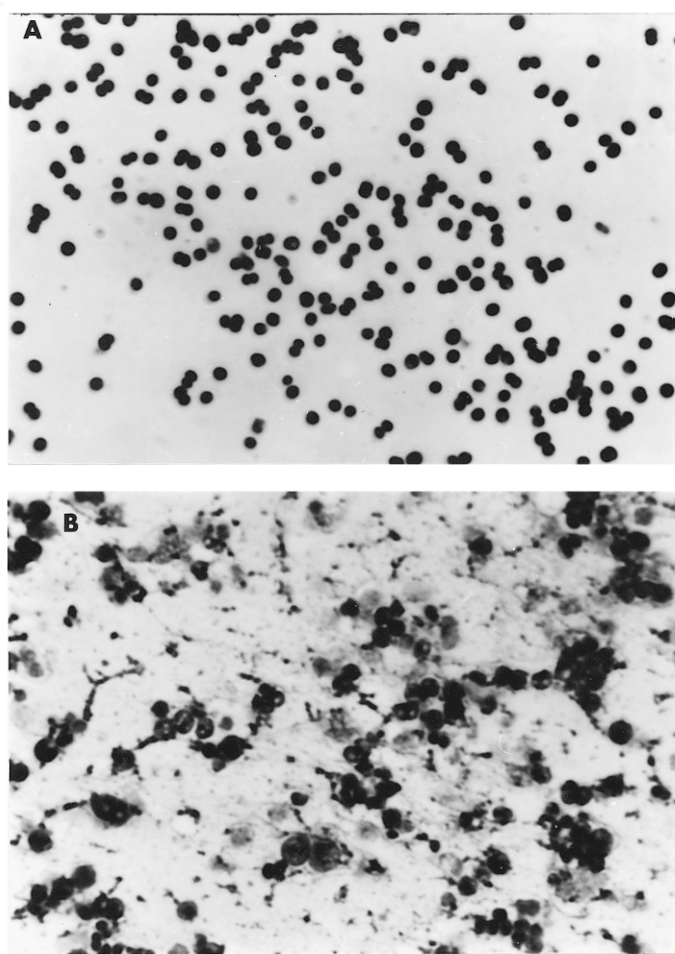


FIGURE BXII.α.26. Light microscopic photographs of *Craurococcus roseus* demonstrating the effect of divalent cations on cell structure when cells are suspended in aqueous solution. (A) Cells suspended in sterilized 25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. (B) Cells suspended in sterilized cation free aqueous solution. (Courtesy of Y. Nishimura.)

grown in NPG medium, and 0.04 nmol/mg dry cell weight when grown in NPGC medium (Saitoh and Nishimura, 1996). Absorption difference spectra (illuminated minus unilluminated) show photochemical activity similar to that found in anaerobic and other aerobic anoxygenic phototrophic bacteria. Carotenoids purified by chromatographic procedures include large amounts of carotenoid acids similar to *Pseudomonas radiosa*, and smaller amounts of spirilloxanthin.

The cellular fatty acid composition of *C. roseus* consists of large amounts of octadecanoic acid and small amounts of octadecadienoic acid, 2-hydroxyoctadecanoic acid, 3-hydroxytetradecanoic acid, and 3-hydroxyoctadecanoic acid. Ubiquinone is the only quinone type detected. The major ubiquinone detected is Q-10 (89–96%) and ubiquinones Q-7, Q-8, and Q-9 are present in smaller quantities. Cellular lipids consist of phospholipids of phosphatidylcholine, phosphatidylethanolamine, bisphosphatidylglycerol, and phosphatidylglycerol.

C. roseus grows heterotrophically under aerobic conditions. Cultures hydrolyze Tweens 20, 40, 60, and 80, and can utilize several substrates as sole carbon and energy sources for heterotrophic growth. Best growth occurs on D-xylose, fumarate, and L-glutamate. Other carbon sources include glycerol, D-fructose, D-fucose, D-galactose, D-glucose, D-arabinose, L-rhamnose, D-ribose, DL-lactate, malonate, pyruvate, gluconate, and L-malate. *C. roseus* does not show gelatinase or amylase activity.

C. roseus is an obligate aerobe, no growth occurs anaerobically under either illuminated or dark conditions. Light at intensity of about 2000 lux has not been shown to stimulate aerobic growth.

Cells do not require salt for growth, and do not grow in media supplemented with 0.4% NaCl. Cells are susceptible to chloramphenicol, penicillin, streptomycin, and tetracycline, and are resistant to polymixin B. Nicotinic acid and pantothenate are required as growth factors.

C. roseus grows at a mesophilic temperature range between 18°C and 37°C, and optimum growth occurs between 28°C and 32°C. The pH range is narrow, with growth occurring only between pH 7.2 and 8.0. Optimal pH for growth is 7.6.

ENRICHMENT AND ISOLATION PROCEDURES

C. roseus was isolated on a 100-fold dilution of nutrient agar media supplemented with 1.5% agar, from soils found in a parking lot in Nerima Ward, Tokyo.

MAINTENANCE PROCEDURES

Cells are maintained on NPG agar medium at pH 8.0. Long-term storage procedures have not been described.

DIFFERENTIATION OF THE GENUS *CRAUROCOCCLUS* FROM OTHER GENERA

The genus *Craurococcus* can be differentiated from other known genera of obligately aerobic phototrophic bacteria based on several characteristics. In a phylogenetic tree derived from 16S rRNA gene sequences, *Craurococcus* forms, with the genus *Paracraurococcus*, a separate line of descent within the *Alphaproteobacteria*. This organism can be differentiated from its closest relatives based on color (yellowish-red to pink colonies) as well as specific absorption spectra characteristics. *C. roseus* is capable of growth over very narrow pH, temperature, and salinity ranges (Table BXII.α.23).

Craurococcus has an unusual requirement for divalent cations (Mg^{2+} or Ca^{2+}) when suspended in aqueous solution. Cell lysis occurs if these divalent cations are not present.

TAXONOMIC COMMENTS

The 16S rDNA sequence of *C. roseus* showed that the genus *Craurococcus* is most closely related to the genus *Paracraurococcus* and that this line falls within a cluster formed by the genera *Roseococcus*, *Rhodospila*, and *Acidiphilium*. The position of *Craurococcus* is a branch between the genus *Paracraurococcus* and the other members of the cluster, within the *Alphaproteobacteria*.

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.
- Yurkov, V.V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

List of species of the genus *Craurococcus*

1. ***Craurococcus roseus*** Saitoh, Suzuki and Nishimura 1998, 1045^{VP}

ro.se'us. L. adj. *roseus* rose-colored, pink, pink bacterium.

Gram negative, forms pink colonies when grown on agar medium. Cells are coccoid, 0.8–2.0 µm in diameter, non-

motile. Cells contain Bchl *a* organized into reaction center and light-harvesting complexes, giving *in vivo* absorption spectrum peaks at 800 and 872 nm, respectively. Obligately aerobic chemoorganoheterotroph. The best growth substrates are D-xylose, fumarate and L-glutamate. Other

TABLE BXII.α.23. Distinguishing characteristics of *Craurococcus roseus*, and closely related *Paracraurococcus ruber*^a

Characteristic	<i>C. roseus</i>	<i>P. ruber</i>
Cell shape and size	Coccoid, 0.8–2.0 µm	Coccoid, 0.8–1.5 µm
Color	Pink	Red
Bchl <i>a</i> peaks (nm)	800, 872	802, 856
Growth at 42°C	+	–
Hydrolysis of:		
Tween 80	–	+
Starch	W	–
Gelatin	–	–
Requirement for divalent cations in aqueous suspension	+	+
Requirement for divalent cations in liquid culture	–	+
Growth at:		
pH 6.8	+	–
pH 8.0	+	+

^aSymbols: +, positive; –, negative, W, weak.

growth substrates are glycerol, D-fructose, D-fucose, D-galactose, D-glucose, D-arabinose, L-rhamnose, D-ribose, DL-lactate, malonate, pyruvate, gluconate, and L-malate. Hydrolyses Tween 20, 40, 60, and 80, but does not hydrolyze starch or gelatin. No growth on D-mannitol, D-sorbitol, methanol, ethanol, 1,2-propanediol, kerosene, acetate, butyrate, citrate, formate, glycolate, propionate, succinate, phthalate, *p*-hydroxybenzoate, and benzoate.

Optimal growth occurs at pH 7.5 and temperature 28–32°C, NaCl is not required. Incapable of growth at NaCl

concentrations of 0.4% and higher. Divalent cations are required for growth. Exhibits oxidase and catalase activity. Reduces nitrate to nitrite. Accumulates poly- β -hydroxybutyrate. Resistant to polymixin B. Susceptible to chloramphenicol, penicillin, streptomycin, and tetracycline. Habitat: soil from a parking lot located in Nerima Ward, Tokyo, Japan.

The mol% G + C of the DNA is: 70.5 (HPLC).

Type strain: NS130, CIP 105707, JCM 9933.

GenBank accession number (16S rRNA): D85828.

Genus VIII. *Gluconacetobacter* Yamada, Hoshino, and Ishikawa 1998b, 32^{VP} (Effective publication: Yamada, Hoshino, and Ishikawa 1997, 1249)*

MARTIN SIEVERS AND JEAN SWINGS

Glu.con.a.ce.to.bac'ter. M.L. n. *acidum*, *gluconicum* gluconic acid; M.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod; M.L. masc. n. *Gluconacetobacter* gluconate-vinegar rod.

Cells **ellipsoidal to rod shaped**, straight or slightly curved, 0.6–1.2 \times 1.0–3.0 μ m, occurring singly, in pairs, or in short chains. **Motile or nonmotile; if motile, the flagella are peritrichous**. Endospores are not formed. Gram negative. **Obligately aerobic**; metabolism is strictly respiratory with oxygen as the terminal electron acceptor. Never fermentative. Some strains produce thick pellicle-forming colonies. Catalase positive and oxidase negative. Absence of gelatin liquefaction, indole production, and H₂S formation. **Oxidize ethanol to acetic acid. Overoxidation of acetate to CO₂ and H₂O depends upon acetate concentration in the medium**. The best carbon sources for growth are ethanol, glucose, and acetate. Acids are formed from glucose and ethanol. Species differ in respect to growth behavior on media with high acetate or glucose concentrations. Optimum temperature is 30°C. **The pH optimum for growth is 2.5–6.0. Possesses ubiquinone of the Q-10 type as major quinone**. The predominant fatty acid in *Gluconacetobacter* is the C_{18:1}_{ω7} straight-chain unsaturated acid. ***Gluconacetobacter* species occur in vinegar, tea fungus, sugarcane, mealy bug, flowers, and fruits, and can cause infections in beverages and spirituous liquors**. *Gluconacetobacter* is not known to have any pathogenic effect toward humans and animals.

The mol% G + C of the DNA is: 55–67.

Type species: Gluconacetobacter liquefaciens (Asai 1935) Yamada, Hoshino and Ishikawa 1998b, 327 (Effective publication: Yamada, Hoshino and Ishikawa 1997, 1250) (*Acetobacter liquefaciens* (Asai 1935) Gosselé, Swings, Kersters, Pauwels, and De Ley 1983c, 896; *Acetobacter aceti* subsp. *liquefaciens* De Ley and Frateur 1974, 277; "*Gluconobacter liquifaciens*" Asai 1935, 610.)

FURTHER DESCRIPTIVE INFORMATION

Based on partial base sequences in positions 1220–1375 (*E. coli* numbering system, Brosius et al., 1981) of 16S rRNA genes, the *Gluconacetobacter* species that have Q-10 clustered remotely from the *Acetobacter* and *Gluconobacter* species (Yamada et al., 1997). The levels of overall 16S rRNA sequence similarity between members within the Q-10 equipped *Gluconacetobacter* species are above 96.5%, whereas those between all of the *Acetobacter* and *Glucon-*

acetobacter species are above 94.0%. The Q-10 equipped *Gluconacetobacter* species formed a cluster closely related to the species of *Acetobacter* in the phylogenetic trees based on complete 16S rRNA sequences (Sievers et al., 1994a). Members of the genus *Acetobacter* have many characteristics in common with *Gluconacetobacter*. *G. europaeus*, *G. intermedius*, *G. oboediens*, *G. entanii*, and *A. pomorum* were isolated from vinegar and share the same ecological niches.

Gluconacetobacter can only be differentiated from *Acetobacter* by the determination of the ubiquinone type. The determination of the ubiquinone type is carried out by the method of reversed-phase paper chromatography (Yamada et al., 1969). Reversed-phase HPLC is used to determine ubiquinones quantitatively (Tamaoka et al., 1983; Franke et al., 1999).

Growth on mannitol agar and acid formation from mannitol occurred in both genera *Gluconacetobacter* and *Acetobacter*. Growth on and acid formation from carbon sources are not necessarily correlated features. Fewer than 10% of the *Acetobacter* strains produce acid from D-mannitol. On the other hand, D-mannitol supported growth for most strains of acetic acid bacteria.

Cultivation media *Gluconacetobacter* species, like *G. diazotrophicus*, *G. hanseni*, and *G. liquefaciens*, grow well on mannitol medium (MYP).¹ *Gluconacetobacter sacchari* strains grow well on GYC medium.² *G. xylinus* strains do not develop on MYP medium and should be cultivated on GY medium.³ *G. europaeus* requires acetic acid for growth and was cultivated on modified AE medium (Entani et al., 1985).⁴ *G. intermedius* grows well on MRS medium developed for lactobacilli (de Man et al., 1960), GY medium, or AE medium. *G. johannae* and *G. azotocaptans* were cultivated on GJA medium.⁵

1. MYP medium (g/l distilled water): D-mannitol, 25.0; yeast extract, 5.0; peptone, 3.0; and agar, 12.0.

2. GYC medium (g/l distilled water): D-glucose, 100.0; yeast extract, 10.0; calcium carbonate, 20.0; and agar, 20.0.

3. GY medium (g/l distilled water): D-glucose, 100.0; yeast extract, 10.0; and agar, 12.0. (A concentrated glucose solution was autoclaved separately to avoid Maillard reactions.)

4. AE medium (per liter distilled water): yeast extract, 3.0 g; peptone, 4.0 g; D-glucose, 7.5 g; agar, 8.0 g; acetic acid, 30 ml (v/v); and ethanol (99.8%), 30 ml. The ethanol and acetic acid are added after sterilization of the basal medium.

5. GJA medium [medium 920 (per liter of distilled water)]: yeast extract, 2.7; D-glucose, 2.7; D-mannitol, 1.8; MES buffer (Sigma), 4.4; K₂HPO₄, 4.81; KH₂PO₄, 0.65; and agar, 15.0 (final pH 6.7).

*Editorial Note: Yamada et al. (1997) proposed the elevation of the subgenus *Gluconacetobacter* (Yamada and Kondo, 1984) to the generic level, based on partial base sequences of the 16S rRNA and the ubiquinone system. The name *Gluconacetobacter* has been corrected to *Gluconacetobacter* in accordance with Rule 61 of the International Code of Nomenclature of Bacteria.

ENRICHMENT AND ISOLATION PROCEDURES

LGI medium is used for enrichment of *G. diazotrophicus*. For isolation of *G. diazotrophicus* from sugarcane roots and stems, acetic LGI agar plates (pH 4.5) supplemented with yeast extract (0.05 g/l) and cycloheximide (0.15 g/l) were used (Fuentes-Ramírez et al., 1993). Roots and stems were washed in sterile tap water, macerated in a blender, and for inoculation, dilutions in sugar solution (5% cane sugar in H₂O) were prepared. The acetic LGI medium consists of (quantities per liter): K₂HPO₄, 0.2 g; KH₂PO₄, 0.6 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.02 g; Na₂MoO₄·2H₂O, 0.002 g; FeCl₃·6H₂O, 0.01 g; bromothymol blue (0.5% solution in 0.2 N KOH), 5 ml; crystallized cane sugar, 100 g; agar, 8.0 g; final pH adjusted to 4.5 with acetic acid (Cavalcante and Döbereiner, 1988).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *GLUCONACETOBACTER*

The differentiation of the species of the genera *Gluconacetobacter*, *Acidomonas*, and *Asaia* is given in Table BXII.α.24.

List of species of the genus *Gluconacetobacter*

1. ***Gluconacetobacter liquefaciens*** (Asai 1935) Yamada, Hoshino and Ishikawa 1998b, 327^{VP} (Effective publication: Yamada, Hoshino and Ishikawa 1997, 1250) (*Acetobacter liquefaciens* (Asai 1935) Gosselé, Swings, Kersters, Pauwels, and De Ley 1983c, 896; *Acetobacter aceti* subsp. *liquefaciens* De Ley and Frateur 1974, 277; "*Gluconobacter liquifaciens*" Asai 1935, 610.)

li.que.fa'ci.ens. L. v. *liquefacio* to liquefy; L. part. adj. *liquefaciens* liquefying.

Colonial and cell morphology are as described for the genus. Motile by peritrichous flagella. 2-Keto-, 2,5-diketo-, and sometimes also 5-ketogluconic acid are synthesized from D-glucose. The majority of the strains produce γ-pyrones from D-glucose and D-fructose. Ketogenesis in *G. liquefaciens* occurs with glycerol, D-mannitol, and sorbitol. Acid is produced from ethanol, *n*-propanol, *n*-butanol, D-mannose, and D-glucose. Most strains are able to grow on *n*-propanol, ethanediol, glycerol, *meso*-erythritol, D-mannitol, D-galactose, D-fructose, D-glucose, Ca-D-gluconate, Ca-D,L-glycerate and Na-D,L-lactate. All strains are able to utilize ammonium as a sole source of nitrogen with ethanol as a carbon source, even without growth factors. Few strains do not require growth factors in the presence of D-mannitol. *G. liquefaciens* causes pink disease in pineapple fruits due to the production of 2,5-diketogluconic acid (Gosselé and Swings, 1986).

The mol% G + C of the DNA is: 62–65 (*T_m*).

Type strain: ATCC 14835, DSM 5603, IAM 1834, IFO 12388.

GenBank accession number (16S rRNA): X75617.

2. ***Gluconacetobacter azotocaptans*** Fuentes-Ramírez, Bustillos-Cristales, Tapia-Hernández, Jiménez-Salgado, Wang, Martínez-Romero and Caballero-Mellado 2001, 1312^{VP} *a.zo.to.cap'tans*. N.L. adj. *azotocaptans* nitrogen-catching.

G. azotocaptans was isolated from the rhizospheres of coffee plants. Cells are straight rods with rounded ends, approximately 1.6–2 × 0.5–0.6 μm, occurring singly or in pairs. Cells are motile by peritrichous flagella. Colonies on potato agar with sugarcane are beige or very light-brownish.

MAINTENANCE PROCEDURES

Strains of *Gluconacetobacter* can be maintained for 2–3 weeks at 4°C on agar media used for cultivation. *Gluconacetobacter* strains, with the exception of *G. europaeus*, can be frozen and kept at –75°C in the presence of 24% (v/v) glycerol. This maintenance procedure is also possible for *G. intermedius* grown in MRS broth. *Gluconacetobacter* survives lyophilization. Freeze-drying is possible under controlled conditions also for *G. europaeus*, e.g., the type strain of *G. europaeus* DSM 6160. Another reliable method is preservation of *Gluconacetobacter* strains under liquid nitrogen. For preservation of *Gluconacetobacter* isolates from vinegar, cells at the end of the logarithmic growth phase cultivated in modified AE broth were immediately centrifuged at 3000 × g at –10°C. They were resuspended and concentrated in ice-cold 20% malt extract and frozen in liquid nitrogen (Sokollek et al., 1998a).

Strains fix molecular nitrogen microaerophilically (Fuentes-Ramírez et al., 2001). Strains grow on D-glucose or sucrose. No growth on D-mannitol, glycerol, and D-xylose. Strains grow on 0.5% ethanol but not with methanol. *G. azotocaptans* can be differentiated from *G. johannae* by RFLP of PCR-amplified 16S rDNA with *RsaI* generating fragments of 504, 404, 246, 159, and 134 bp, respectively (Fuentes-Ramírez et al., 2001).

The mol% G + C of the DNA is: 64 (*T_m*).

Type strain: ATCC 700988, DSM 13594.

GenBank accession number (16S rRNA): AF192761.

3. ***Gluconacetobacter diazotrophicus*** (Gillis, Kersters, Hoste, Janssens, Kroppenstedt, Stephan, Teixeira, Döbereiner and De Ley 1989) Yamada, Hoshino and Ishikawa 1998b, 327^{VP} (Effective publication: Yamada, Hoshino and Ishikawa 1997, 1250) (*Acetobacter diazotrophicus* Gillis, Kersters, Hoste, Janssens, Kroppenstedt, Stephan, Teixeira, Döbereiner and De Ley 1989, 362.)

di.a.zo.tro'phi.cus. M.L. masc. adj. *diazotrophicus* one that feeds on dinitrogen.

Straight rods with rounded ends. Motile by lateral or peritrichous flagella. Produces brown water-soluble pigments on D-glucose, yeast extract medium. Dark brown colonies are formed on potato agar supplemented with 10% sucrose. Forms 2-ketogluconic acid and 2,5-diketogluconic acid from glucose. The optimal growth pH is 5.5. High concentrations (10%) of sucrose are used for isolation of *G. diazotrophicus* strains and a medium composed of 2.5% mannitol, 0.5% yeast extract, and 0.3% peptone is suitable for cultivation.

Isolated from roots, stems, and leaves of sugarcane (Gillis et al., 1989), and coffee (Jiménez-Salgado et al., 1997) and, in addition, has been recovered from sweet potato, Cameroon grass (Caballero-Mellado et al., 1995), and mealy bugs, which act as a vector for carrying *G. diazotrophicus* to the leaf tissue of sugarcane (Ashbolt and Inkerman, 1990). *G. diazotrophicus* does not survive in the soil without the presence of host plants and is growing within a low pO₂ environment, which is necessary for the expression and

TABLE BXII.α.24. Differentiating features among species of *Gluconacetobacter*, *Acidomonas*, and *Asaia*^a

Characteristics	<i>G. liquefaciens</i>	<i>G. azolocapnans</i>	<i>G. diazotrophicus</i>	<i>G. entanii</i>	<i>G. europaeus</i>	<i>G. hansenii</i>	<i>G. intermedius</i>	<i>G. johannae</i>	<i>G. oboediens</i> ^b	<i>G. sacchari</i>	<i>G. xylinus</i>	<i>Acidomonas methanolica</i>	<i>Asaia bogorensis</i>	<i>Asaia siamensis</i>
Growth on 3% (v/v) ethanol in the presence 5–8% acetic acid	–	–	–	+	+	–	+	–	+	–	–	–	–	–
Requirement of acetic acid for growth	–	–	–	+	+	–	–	–	–	–	–	–	–	–
Growth only in the presence of acetic acid and ethanol and glucose ^c	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Growth on the medium of Carr and Passmore	+	nd	+	–	–	+	(+)	nd	nd	+	(+)	+	nd	nd
Growth on methanol	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Formation from D-glucose of:														
5-ketogluconic acid	d	nd	–	–	d	d	–	nd	–	+	+	–	+	+
2,5-diketogluconic acid	+	nd	+	–	–	–	–	nd	–	+	–	–	–	–
Growth on carbon source ethanol	+	+	+	+	+	d	+	+	+	+	d	+	– or w	– or w
Growth in the presence of 30% (w/v) D-glucose	–	+	+	–	–	–	+	+	+	+	–	–	nd	nd
Growth in the presence of 30% (w/v) D-glucose with formation of ≥130 g/l gluconic acid	nd	nd	nd	nd	nd	nd	nd	nd	+	nd	nd	nd	nd	nd
Growth on 0.01% malachite-green agar	+	nd	–	nd	–	nd	nd	nd	nd	+	–	nd	nd	nd
N ₂ fixation	–	+	+	–	–	–	–	+	–	–	–	–	–	–
Cellulose formation	–	–	–	–	+ or –	–	+	–	–	–	+	–	–	–
Ubiquinone type	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
Mol% G + C content	62–65	64	61–63	58	56–58	58–63	62	58	60	62–67	55–63	62	59–61	59–60
<i>Preferred identification method besides ethanol oxidation and overoxidation of acetic acid:</i>														
Phenotypically ^d				+	+		+			+	+	+	+	+
Genotypically by:														
16S rRNA sequence ^e	+		+			+								
ARDRA ^f		+						+						

^aSymbols: +, 90% or more of the strains positive; w, weakly positive reaction; d, 11–89% of the strains positive; –, 90% or more of the strains negative; nd, not determined.^bDNAs from the type strains of *G. intermedius* and *G. oboediens* showed species-level similarity of 76% among each other.^cSum of acetic acid and ethanol has to exceed 6% (Schüller et al., 2000).^dData given under the description of the species.^eData from Sievers et al. (1994a).^fAmplified rDNA restriction analysis (Fuentes-Ramírez et al., 2001).

functioning of the nitrogenase (James and Olivares, 1997). The mechanisms of infection and colonization of sugarcane by *G. diazotrophicus* are described by James and Olivares (1997).

G. diazotrophicus is able to fix nitrogen in the presence of nitrates and at low pH values (Stephan et al., 1991; Burris, 1994) and seems best adapted to the sugarcane environment, since the nitrogenase activity is stimulated in mixed cultures (Cojho et al., 1993). Strains fix molecular nitrogen under microaerobic conditions. Sucrose concentration of 10% has a positive effect on nitrogenase activity protecting nitrogenase against inhibition by oxygen (Reis and Döbereiner, 1998). During N_2 -fixation by *G. diazotrophicus*, activity of glucose dehydrogenase is enhanced and cytochrome a_1 (= cytochrome *ba*) is expressed as terminal oxidase (Flores-Encarnación et al., 1999). Most *G. diazotrophicus* strains produce levan as an exopolysaccharide that is synthesized by levansucrase (Hernández et al., 1995). The bacterium grows in the presence of 30% glucose and exhibits high rates of gluconic acid formation (Attwood et al., 1991). *G. diazotrophicus* grows well on sucrose, D-glucose, D-fructose, D-gluconate, and polyols like D-mannitol, D-sorbitol, and glycerol. The lack of growth on C4-dicarboxylates such as succinate, fumarate, and malate is due to the absence of a transport system for dicarboxylates in *G. diazotrophicus* (Alvarez and Martínez-Drets, 1995; Ureta et al., 1995). The lipopolysaccharide (LPS) of *G. diazotrophicus* was characterized by Fontaine et al. (1995) and contains rhamnose, mannose, and galactose as monosaccharide constituents in all investigated strains.

The mol% G + C of the DNA is: 61–63 (T_m).

Type strain: ATCC 49037, PAL 5, DSM 5601, LMG 7603.

GenBank accession number (16S rRNA): X75618.

4. **Gluconacetobacter entanii** Schüller, Hertel and Hammes 2000, 2019^{VP}

en.ta'ni.i. L. gen. *entanii* of Entani, the name honors Etsuzo Entani, a Japanese microbiologist.

Cells are ellipsoidal to rod shaped, straight, or slightly curved, $0.8\text{--}1.2 \times 1.3\text{--}1.6 \mu\text{m}$, nonmotile, occurring singly, in chains, and mainly in pairs. Colonies are round, regular, umbonate, soft, glossy, and with a diameter of 1–2 mm on AE agar (Schüller et al., 2000).

G. entanii requires acetic acid in addition to ethanol and glucose for growth. Growth occurs only at total concentrations (sum of acetic acid and ethanol) exceeding 6.0%. At this concentration, *G. entanii* is not able to oxidize acetic acid further to CO_2 and H_2O . Oxidizes ethanol to acetic acid. Growth on 3% (v/v) ethanol in the presence of up to 11% acetic acid (v/v). Gluconate, glycerol, methanol, and lactate are not used as carbon source. The ability to utilize sorbitol and mannitol is strain dependent. No formation of ketogluconic acids from glucose. No formation of cellulose. Good growth on glucose, fructose, and sucrose in AE broth (4a/3e). AE broth (4a/3e) is composed of 0.5% glucose, 0.2% yeast extract, 0.3% peptone, 4% (w/v) acetic acid, and 3% ethanol (v/v) (Entani et al., 1985; Sievers et al., 1992; Sokollek and Hammes, 1997). *Gluconacetobacter entanii* is closely related to *G. hansenii* based on 16S rRNA sequence similarity. *G. entanii* can be distinguished from *G. hansenii* by its ability to grow on 3% (v/v) ethanol in the presence of 4–8% (w/v) acetic acid. *G. entanii* requires, in

contrast to *G. europaeus*, ethanol as well as acetate and glucose for growth.

The mol% G + C of the DNA is: 58 (T_m).

Type strain: DSM 13536, LTH 4560.

GenBank accession number (16S rRNA): AJ251110.

5. **Gluconacetobacter europaeus** (Sievers, Sellmer and Teuber 1992) Yamada, Hoshino and Ishikawa 1998b, 327^{VP} (Effective publication: Yamada, Hoshino and Ishikawa 1997, 1250) (*Acetobacter europaeus* Sievers, Sellmer and Teuber 1992, 656.)

eu.ro.pae'us. L. adj. masc. *europaeus* occurring in or coming from Europe.

Cells are rod shaped, straight, $0.7 \times 2 \mu\text{m}$, occurring singly or in pairs. Mobility and flagella not observed. Pale colonies, no pigments produced. Strains have an absolute requirement of acetic acid for growth. Growth occurs on AE medium in a relative humidity of 92–97%. *G. europaeus* growing on AE medium utilizes glucose and ethanol simultaneously. Cells die rapidly without an oxygen supply (Hitschmann and Stockinger, 1985). Catalase positive, oxidase negative. Acetate is oxidized to CO_2 and H_2O below an acetate concentration of 6%. Glucose is oxidized to 5-ketogluconate or 2-ketogluconate. Some strains produce cellulose/acetan.

Acetic acid bacteria produce hopanoids acting as membrane stabilizers (Rohmer et al., 1984; Ourisson et al., 1987). *G. europaeus* contains novel series of methylhopanoids with an additional methyl group at position C31 (Simonin et al., 1994).

Gluconacetobacter europaeus has been isolated from vinegar fermentations and is used in submerged cultures and trickling generators for industrial production of vinegar.

G. europaeus strains can be freeze-dried under controlled conditions and reactivated. Preservation of strains at -70°C in the presence of 25% glycerol and revival of these strains is still difficult.

The overall 23S rDNA sequence similarity values of *G. europaeus* type strain with the type strains of *G. intermedius* and *G. xylinus* are 99.2% and 98.9%, respectively.

The mol% G + C of the DNA is: 55–58 (T_m).

Type strain: ATCC 51845, DSM 6160.

GenBank accession number (16S rRNA): Z21936.

Additional Remarks: The accession number of the 23S rDNA of *G. europaeus* type strain is X89771 (Boesch et al., 1998).

6. **Gluconacetobacter hansenii** (Gosselé, Swings, Kersters, Pauwels and De Ley 1983c) Yamada, Hoshino and Ishikawa 1998b, 327^{VP} (*Acetobacter hansenii* Gosselé, Swings, Kersters, Pauwels and De Ley 1983c, 896.)

han.se'ni.i. M.L. n. *hansenii* of E.C. Hansen, a Danish microbiologist, well known for his study on the acetic acid bacteria.

The cell morphology and colonial characteristics are as described for the genus. Ketogenic toward glycerol, D-mannitol, and sorbitol. Most strains produce 2-ketogluconic acid and 5-ketogluconic acid from D-glucose. Acid is produced from ethanol, *n*-propanol, *n*-butanol, D-mannose, and D-glucose. Strains of *G. hansenii* are unable to grow on ethanol, if ethanol is the sole carbon source and yeast extract is absent. Strains require growth factors.

The mol% G + C of the DNA is: 58–63 (T_m).

Type strain: ATCC 35959, DSM 5602, LMG 1527, NCIMB 8746.

GenBank accession number (16S rRNA): X75620.

7. ***Gluconacetobacter intermedius*** (Boesch, Trček, Sievers and Teuber 1998) Yamada 2000, 2226^{VP} (*Acetobacter intermedius* Boesch, Trček, Sievers and Teuber 1998, 1083.) *in.ter.me'di.us.* L. adj. masc. *intermedius* in the middle between.

Cells are rod shaped, straight, $0.7 \times 2 \mu\text{m}$, occurring singly or in pairs. Motility and flagella not observed. Pale colonies, no pigments produced. Catalase positive, oxidase negative. Acetate is oxidized to CO_2 and H_2O below an acetate concentration of 6%. *G. intermedius* strains grow with or without acetic acid. Growth occurs on AE medium in the presence of 3% (v/v) ethanol and up to 8% (v/v) acetic acid. Eleven of 13 strains of *G. intermedius* grow on GY medium and all strains show strong growth on MRS medium. No oxidation of glucose to 5-ketogluconate. Formation of dihydroxyacetone from glycerol. Most strains do not utilize sucrose as carbon source. Strains produce cellulose/acetan. *Gluconacetobacter intermedius* occurs in tea fungus beverages (Sievers et al., 1995b), spirit vinegar, and cider vinegar fermentations. The type strain TF2 (DSM 11804) was isolated from a commercially available tea fungus beverage (Kombucha) in Switzerland. Kombucha is a refreshing beverage obtained from fermentation of sugared tea with a symbiotic culture of *G. intermedius* and yeasts. The effects of Kombucha on human health are discussed by Dufresne and Farnworth (2000). *G. intermedius* produces a thick cellulose/acetan matrix under static conditions, which covered the surface of the beverage and keeps the cells in close contact with the atmosphere. The overall 23S rDNA sequence similarity values of *G. intermedius* type strain with the type strains of *G. europaeus* and *G. xylinus* are 99.2% and 99.0%, respectively.

An oligonucleotide probe based on the 23S rDNA of *G. europaeus* (23seu: 5'-AATGCGCCAAAAGCCGGAT-3') developed for *G. europaeus* hybridizes with *G. europaeus* strains and with the following *G. xylinus* strains: LMG 25, ATCC 23768, and ATCC 10245. The probe does not hybridize with *G. intermedius* strains and is thus useful for the differentiation of *G. intermedius* from *G. europaeus* (Boesch et al., 1998).

The mol% G + C of the DNA is: 62 (T_m).

Type strain: TF2, DSM 11804.

GenBank accession number (16S rRNA): Y14694.

Additional Remarks: The accession number of the 23S rDNA sequence of the *G. intermedius* type strain is Y14680.

8. ***Gluconacetobacter johannae*** Fuentes-Ramírez, Bustillos-Cristales, Tapia-Hernández, Jiménez-Salgado, Wang, Martínez-Romero and Caballero-Mellado 2001, 1312^{VP} *jo.han'nae.* N.L. gen. n. *johannae* of Johanna, in honor of the Brazilian microbiologist, Johanna Döbereiner, who isolated *G. diazotrophicus* as the first nitrogen-fixing species of the genus *Gluconacetobacter*.

G. johannae was isolated from the rhizospheres of coffee plants. Cells are straight rods with rounded ends, approximately $1.5\text{--}1.9 \times 0.5\text{--}0.6 \mu\text{m}$, occurring singly, in pairs, or in short chains. Cells are motile by peritrichous flagella. Colonies on potato agar with sugarcane are brown. Strains fix molecular nitrogen microaerophilically (Fuentes-Ramí-

rez et al., 2001). Strains grow in 30% D-glucose or sucrose. Slight to no growth on D-mannitol and glycerol. Strains grow on D-xylose as carbon source. Strains grow on 0.5% ethanol but not with methanol. *G. johannae* ATCC 700987 showed 45% DNA relatedness to the type strain of *G. azotocaptans*. *G. johannae* can be differentiated from *G. azotocaptans* by RFLP of PCR-amplified 16S rDNA with *RsaI* generating fragments of 504, 405, 403, and 135 bp, respectively (Fuentes-Ramírez et al., 2001).

The mol% G + C of the DNA is: 58 (T_m).

Type strain: ATCC 700987, DSM 13595.

GenBank accession number (16S rRNA): AF111841.

9. ***Gluconacetobacter oboediens*** (Sokollek, Hertel, Hammes 1998b) Yamada 2000, 2226^{VP} (Sokollek, Hertel, Hammes 1998b, 939.)

ob.oe.di'ens. L. adj. *oboediens* obedient.

G. oboediens DSM 11826 is phenotypically and genotypically similar to *G. intermedius* in respect to growth with and without acetic acid, and 16S rRNA sequence similarity is 100%. The type strains of *G. intermedius* and *G. oboediens* showed 76% DNA relatedness among each other (Sievers and Schumann, unpublished). Based on the DNA-DNA hybridization data and phenotypic similarities, it is obvious that *G. oboediens* is synonymous with *G. intermedius*. *G. oboediens* is able to grow on 3% (v/v) ethanol in the presence of up to 8% (w/w) acetic acid. No absolute requirement of acetic acid for growth. Growth in the presence of 30% (w/v) glucose with a strong formation of gluconic acid ($\geq 130 \text{ g/l}$). Good growth on glucose, fructose, ribose, glycerol, and sucrose on yeast extract peptone agar. Cellulose is not formed.

Gluconacetobacter oboediens, type strain DSM 11826, was isolated from a submerged red wine vinegar fermentation at a factory in the southern part of Germany.

The mol% G + C of the DNA is: 60 (T_m).

Type strain: DSM 11826, LTH 2460.

GenBank accession number (16S rRNA): AJ001631.

10. ***Gluconacetobacter sacchari*** Franke, Fegan, Hayward, Leonard, Stackebrandt and Sly 1999, 1691^{VP} *sacch.ar'i.* M.L. gen. n. *sacchari* pertaining to the genus *Saccharum*, sugarcane, from which the organism was isolated.

Cells of *G. sacchari* are ellipsoidal to rod shaped, approximately $0.7\text{--}0.9 \times 1.3\text{--}2.2 \mu\text{m}$ in size and occur singly, in pairs, or in short chains. Cells are motile by peritrichous flagella. An electron micrograph of the type strain DSM 12717 showing cellular morphology and flagellation is given in Fig. BXII.α.27 (Franke et al., 1999). *Gluconacetobacter sacchari* was isolated from the leaf sheath of sugarcane and from the pink sugarcane mealy bug *Saccharicoccus sacchari*. The occurrence of *G. sacchari* in mealy bug homogenates was detected by *in situ* hybridization with a Cy3-labeled specific primer targeting the 16S rRNA of *G. sacchari* (Franke et al., 2000).

G. sacchari strains grow in the presence of 0.01% malachite-green agar as does *G. liquefaciens*. To test the ability to grow in the presence of the dye malachite green, the agar plates containing (per liter of distilled water): 0.5 g yeast extract, 3.0 g vitamin-free Casamino acids, 1.0 g D-glucose, 1.0 g mannitol, 1.0 g calcium DL-lactate, 25 g agar, and 0.1 g malachite green (Gosselé et al., 1983a). *G. sacchari* strains grow in the presence of 30% D-glucose. Strains grow

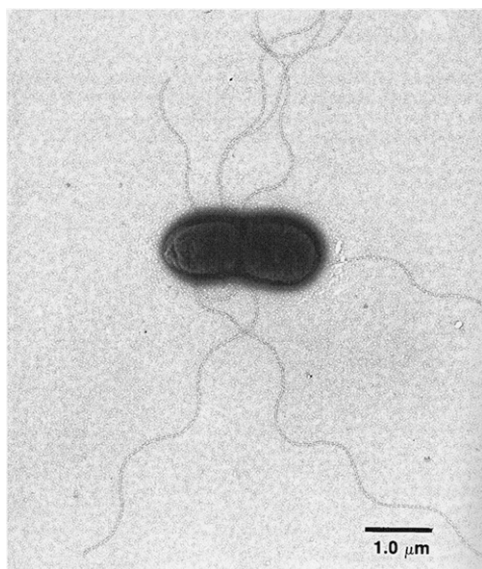


FIGURE BXII.α.27. Electron micrograph of *Gluconacetobacter sacchari* DSM 12717, showing rod-shaped to ellipsoidal cell form and peritrichous flagella. (Reprinted with permission from I.H. Franke et al., International Journal of Systematic and Evolutionary Microbiology 49: 1681–1693, 1999, ©Society of General Microbiology, UK.)

on mannitol agar. Acid is produced from D-glucose, ethanol, and 1-propanol, but not from D-mannitol or sorbitol.

Strains of *G. sacchari* do not grow on the amino acids L-asparagine, L-glycine, L-glutamine, L-threonine, or L-tryptophan with D-mannitol as the carbon source⁶ (Franke et al., 1999). *G. sacchari*, in contrast to *G. liquefaciens*, is not able to utilize these amino acids as the sole nitrogen source with D-mannitol as a carbon source.

Gluconacetobacter sacchari strains are closely related to *G. liquefaciens* by sharing 98.8–99.3% 16S rDNA sequence similarity. The DNA–DNA relatedness of the type strain of *G. sacchari* is 46% with *G. liquefaciens* (Franke et al., 1999). *G. sacchari* does not fix nitrogen in contrast to *G. diazotrophicus*, *G. azotocaptans*, and *G. johannae*.

6. The medium for testing the utilization of L-amino acids as sole nitrogen sources contains (per liter of 0.2 M Tris-maleate buffer, pH 5.4): D-mannitol, 30.0 g; L-amino acid, 1.0 g; salt solution A (per liter of distilled water: KH_2PO_4 , 100 g and K_2HPO_4 , 100 g), 5.0 ml; salt solution B (per liter of distilled water: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 40.0 g; NaCl, 2.0 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.5 g), 5.0 ml; D-biotin, calcium pantothenate, thiamine, folic acid, *p*-aminobenzoic acid, and vitamin B₁₂, 0.001 g each; pyridoxal-HCl, niacin and riboflavin, 0.0015 g each.

The mol% G + C of the DNA is: 62–67 (T_m).

Type strain: DSM 12717, SRI 1794.

GenBank accession number (16S rRNA): AF127407.

11. ***Gluconacetobacter xylinus*** (Brown 1886) Yamada, Hoshino and Ishikawa 1998b, 327^{VP} (Effective publication: Yamada, Hoshino and Ishikawa 1997, 1250) (“*Bacterium xylinum*” Brown 1886; *Acetobacter xylinus* (Brown 1886) Yamada 1984, 270; *Acetobacter aceti* subsp. *xylinus* (Brown 1886) De Ley and Frateur 1974, 277.)*

xy.li' nus. L. adj. *xylinus* woody, derived from; Gr. *xylon* wood.

Cells occurring singly, in pairs, in chains, or in small clusters. Peritrichously flagellated when motile. Synthesize cellulose and acetan. No growth on 3% ethanol in the presence of 5–8% acetic acid. *G. xylinus* strains grow well at D-glucose concentration of 100 g per liter medium. Acids are formed from ethanol, D-glucose, and D-xylose.

Acid formation from sucrose, D-galactose, and production of 5-ketogluconic acid from D-glucose is strain dependent. The type strain of *G. xylinus* does not produce acid from sucrose. The DNA from the type strain of *G. xylinus* showed 69–100% similarity to those from seven *G. xylinus* strains that form acid from sucrose (Tanaka et al., 2000b). *G. xylinus* BPR 2001 (*Acetobacter xylinus* subsp. *sucrofermentans*) differs from *G. xylinus* ATCC 23767 by growth on sucrose, by acid formation from sucrose and by oxidation of lactose (Toyosaki et al., 1995). 16S rRNA sequence similarity of *G. xylinus* ATCC 23767 with *G. xylinus* BPR 2001 (*Acetobacter xylinus sucrofermentans* JCM 9730) is 99.6%. Based on DNA–DNA relatedness, *G. xylinus* and *A. xylinus* subsp. *sucrofermentans* are genetically to homologous to maintain a subspecies under this species level (Tanaka et al., 2000b).

The overall 23S rDNA sequence similarity values of *G. xylinus* type strain with the type strains of *G. intermedius* and *G. europaeus* are 99.0% and 98.9%, respectively.

The mol% G + C of the DNA is: 59–63 (T_m).

Type strain: ATCC 23767, DSM 6513, IFO 15237, NCIMB 11664.

GenBank accession number (16S rRNA): X75619.

Additional Remarks: The accession number of the 23S rDNA of *G. xylinus* is X89812 (Boesch et al., 1998).

*Editorial Note: The name *Acetobacter xylinum* was corrected to *Acetobacter xylinus* by Euzéby (1997) because the epithet *xylinus* as adjective to *Acetobacter* has to be masculine.

Genus IX. *Gluconobacter* Asai 1935, 689^{AL}

MARTIN SIEVERS AND JEAN SWINGS

Glu.co.no.bac' ter. M.L. n. *acidum*, *gluconicum* gluconic acid; M.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod; M.L. masc. n. *Gluconacetobacter* gluconate rod.

Ellipsoidal to rod-shaped cells, 0.5–1.0 × 2.6–4.2 μm, occurring singly and/or in pairs, rarely in chains. The formation of enlarged, irregular cells may occur. Endospores are not formed. **Motile or nonmotile; if motile, the cells have 3–8 polar flagella.** Gram negative. Obligately aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor.

Metabolism is never fermentative. Optimal temperature for growth is 25–30°C. Optimal pH for growth is 5.0–6.0; most strains will grow at pH 3.5. Catalase positive, oxidase negative. Negative for nitrate reduction, gelatin liquefaction, indole production, and H₂S formation. **Oxidize ethanol to acetic acid. Do not oxidize acetate or lactate to CO₂ and H₂O.** *Gluconobacter* strains generally

produce acid during growth on fructose, glucose, xylose, and maltose and tolerate up to 10% glucose. **Ketogenesis occurs from polyalcohols** (for example, dihydroxyacetone from glycerol). **All strains produce 2-ketogluconic acid from D-glucose**, and the majority of strains also form 5-ketogluconic acid. Formation of water-soluble brown pigment is correlated with the production of 2,5-diketogluconic acid and γ -pyrones from D-glucose. Most strains form acid from ethanol, D-mannitol, D-fructose, D-glucose, D-maltose, glycerol, and D-xylose. Few strains produce acid from lactose. The best carbon sources for growth are D-mannitol and D-glucose. **Possesses ubiquinone of the Q-10 type as major quinone**. The predominant fatty acid in *Gluconobacter* is the C_{18:1} ω 7 straight-chain unsaturated acid. **Isolated from sugar-rich environments such as fruits and flowers, honey bees, grapes and wine, palm sap, cocoa wine, cider, beer, and soft drinks.**

The mol% G + C of the DNA is: 52–64.

Type species: *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961, 304 emend. Mason and Claus 1989, 181.

FURTHER DESCRIPTIVE INFORMATION

Metabolism *Gluconobacter* catabolizes D-glucose by the hexose monophosphate pathway. *Gluconobacter* is not able to overoxidize acetic acid to CO₂ and H₂O due to an incomplete tricarboxylic acid cycle, lacking succinate dehydrogenase (Greenfield and Claus, 1972). *Gluconobacter* strains prefer sugar-enriched environments in contrast to *Acetobacter* and *Gluconacetobacter* strains, which prefer alcohol-enriched environments (Asai, 1968).

Vitamin C production *Gluconobacter* species are used for oxidation of D-sorbitol into L-sorbose in the commercial production of vitamin C (ascorbic acid). Vitamin C is industrially produced by the Reichstein method: D-Glucose \rightarrow D-Sorbitol \rightarrow L-Sorbose \rightarrow Diacetonesorbose \rightarrow Diacetone-2-keto-L-gulonic acid \rightarrow L-Ascorbic acid. The process begins with the nonbiological hydrogenation of D-glucose to produce D-sorbitol. The synthesis of L-sorbose from D-sorbitol is carried out biochemically by *Gluconobacter*. The L-sorbose is then converted nonbiologically to L-ascorbic acid. The PQQ-dependent sorbitol dehydrogenase from *G. oxydans* was characterized by Choi et al. (1995a).

Improved methods for vitamin C production that involve the biological formation of 2-keto-L-gulonic acid from D-sorbitol as an intermediate have been developed. The metabolic pathway for biosynthesis of 2-keto-L-gulonic acid from D-sorbitol by *Gluconobacter oxydans* includes L-sorbose as an intermediate: D-Sorbitol \rightarrow L-Sorbose \rightarrow L-Sorbose \rightarrow 2-Keto-L-gulonic acid. The 2-keto-L-gulonic acid is then converted nonbiologically to vitamin C (Hoshino et al., 1990; Saito et al., 1997). Mutagenesis of "*Gluconobacter oxydans*" subsp. *melanogenes* IFO 3293 strain resulted in production of 60 g of 2-keto-L-gulonic acid per liter from 100 g D-sorbitol per liter (Sugisawa et al., 1990). Suppression of the formation of L-idonate from 2-keto-L-gulonic acid is a prerequisite for an improved 2-keto-L-gulonic acid production in *Gluconobacter*. Overexpression of the L-sorbose dehydrogenase and L-sorbose dehydrogenase genes in *G. oxydans* resulted in an improved 2-keto-L-gulonic acid production of the recombinant strain (up to 230%) compared to that of the wild-type strain (Saito et al., 1997). Production of 2-keto-L-gulonic acid from L-sorbose via L-sorbose is catalyzed in *G. oxydans* IFO 3293 by a membrane-bound L-sorbose dehydrogenase and a L-sorbose dehydrogenase located in the cytoplasm. Cloning of the gene coding for the membrane-bound L-sorbose dehydrogenase

from *Gluconacetobacter liquefaciens* IFO 12258 and expression of this gene in *G. oxydans* IFO 3293 revealed a recombinant strain that could carry out the formation of 2-keto-L-gulonic acid by membrane-bound dehydrogenases (Shinjo et al., 1995). *Ketogulonigenium vulgare* DSM 4025 (former classified as *G. oxydans* DSM 4025) oxidizes L-sorbose to 2-keto-L-gulonic acid (Urbance et al., 2001) and produces vitamin C from L-gulonolactone. The enzyme L-gulonolactone dehydrogenase catalyzes this oxidation reaction, has a molecular weight of 110 kDa, and is composed of three subunits (Sugisawa et al., 1995).

Glucose dehydrogenase *Gluconobacter* strains are not known to have any pathogenic effect toward humans or animals but are capable of causing a bacterial rot of apples and pears, which is accompanied by various shades of browning. The bacteria enter the apples through wounds in the cuticula and apple tissue. Strains of *G. oxydans* cause pink disease of pineapple, which is characterized by the formation of pink to deep brown discolorations after heating (e.g., during canning) of the diseased fruit. Glucose dehydrogenase (GDH) is responsible for this fruit discoloration (Cha et al., 1997). GDH catalyzes the oxidation of D-glucose to D-gluconate at the outer surface of the cytoplasmic membrane. D-Gluconate is oxidized by the activity of the D-gluconate-dehydrogenase to 2-ketogluconic acid, which is further converted to 2,5-diketogluconate catalyzed by the 2,5-diketogluconate dehydrogenase (Qazi et al., 1991; Matsushita et al., 1994). 2,5-Diketogluconate may react with natural compounds in the juice to become chromogenic upon heating (Cha et al., 1997). 2,5-Diketogluconate has been also detected in *Gluconacetobacter liquefaciens* strains (Gosselé et al., 1980). Gluconic acid production in *G. oxydans* is mainly the result of the membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase, because the activity of the quinoprotein is 30-fold higher than the activity of the cytoplasmic NADP-dependent GDH (Pronk et al., 1989). The PQQ-dependent GDH from *G. oxydans* consists of 808 amino acids encoded from a 2424-bp DNA sequence. A one-point mutation at nucleotide 2359 has replaced the histidine at position 787 with asparagine in the protein of a *G. oxydans* strain. This single amino acid substitution changes the substrate specificity of the quinoprotein GDH, resulting in the conversion of maltose to maltobionic acid in addition to the glucose oxidation (Cleton-Jansen et al., 1991).

Intact cells of *G. oxydans* with glucose oxidation activity in the late exponential phase of 13 μ mol of glucose/g dry weight per min were used for construction of a biosensor for determination of glucose. The sensitivity of the glucose sensor was 50 nA/mM glucose and the range of the calibration curve was 0–0.8 mM of glucose concentrations (Švitel et al., 1998).

Gluconobacter strains flourish on sugars and are found in sugary niches such as flowers and fruits. *Gluconobacter* is a typical spoiler of soft drinks causing off-flavors in, e.g., orange juice. *Gluconobacter* strains are also found associated with palm trees, the sap of which is fermented to palm wine. *Gluconobacter oxydans* strains have been isolated from grapes and detected as cider- and beer-spoiling organisms (Swings, 1992).

Plasmids and phages Strains of *G. oxydans* typically cannot utilize lactose as carbon source. The lactose transposon Tn951 was conjugally introduced from *E. coli* (RP1::Tn951) to *G. oxydans*. The heterologous expression level of Tn951-encoded β -galactosidase in a *G. oxydans* strain was less than 5% of the fully induced *E. coli* activity (Condon et al., 1991). Transposon Tn5 was introduced in *G. oxydans* ATCC 9937 by conjugation using plasmid

pSUP 2021. Tn5 transposition occurred on the plasmid pVJ1 resulting in a mutant of this strain deficient in glucose dehydrogenase activity (Gupta et al., 1997). The shuttle vector pGE1 (mob, Km^r), which can replicate both in *G. oxydans* and *E. coli*, has been used for cloning the L-sorbose dehydrogenase gene of *Gluconacetobacter liquefaciens* IFO 12258 in *G. oxydans* IFO 3293 to improve the production of 2-keto-L-gulonic acid in the vitamin C manufacturing process (Shinjoh and Hoshino, 1995).

The *G. oxydans* plasmid pGO128 was cloned in pACYC177 and its complete nucleotide sequence was determined (AJ428837). Plasmid pGO128 from *G. oxydans* DSM 3504 is composed of 4340 bp. The analysis of the nucleotide sequence revealed three open reading frames, located on the same strand. ORF1 encodes for a resolvase. The protein encoded by ORF2 is 38% homologous to a virulence-associated protein vapE from the plant pathogen bacterium *Xylella fastidiosa*.

Phage A-1 (Schocher et al., 1979; Jucker and Ettlinger, 1981; Robakis et al., 1985a) and phages GW6210 and JW2040 (Robakis et al., 1985b) from *G. oxydans* have been isolated and characterized. Phage A-1 caused abnormalities in the microbial process of the oxidation of D-sorbitol to L-sorbose by *G. oxydans* (Schocher et al., 1979).

ENRICHMENT AND ISOLATION PROCEDURES

Enrichment of *Gluconobacter* strains present in flowers, fruits, grapes, bees, beer, palm wine, and soft drinks can be achieved using the following medium (g per liter of distilled water): D-glucose, 100.0; yeast extract, 5.0; peptone, 3.0; acetic acid, 1.0; cycloheximide 0.1. After incubation at 30°C for 3–5 d in flasks with shaking, those cultures showing growth are streaked onto plates of a CaCO₃-containing medium, where the colonies dissolve the CaCO₃ (Carr and Passmore, 1979). The medium for the chalk-ethanol test is composed of (per liter of distilled water): glucose, 0.5 g; yeast extract, 5.0 g; peptone, 3.0 g; calcium carbonate, 15.0 g; agar, 12.0 g; and ethanol (99.8%), 15 ml (sterile-filtered and added after sterilization of the basal medium). The overoxidation of acetic acid by *Acetobacter*, *Gluconacetobacter*, and *Acidomonas* strains results in a reprecipitation of CaCO₃. Colonies that dissolve the CaCO₃ without reprecipitation of the CaCO₃ are isolated, further purified on GYC medium¹, and characterized for identification and distinction from *Frateruia*.

MAINTENANCE PROCEDURES

Stock cultures of *Gluconobacter* should be grown on GYC medium, MYP medium,² or DSY medium³ at 28–30°C. The cultures can

be maintained for short-term storage in a refrigerator (4–5°C) for 3–4 weeks. For long-term preservation, *Gluconobacter* can be frozen and kept at –75°C in the presence of 24% (v/v) glycerol. *Gluconobacter* strains can be stored as lyophilized cultures.

DIFFERENTIATION OF THE GENUS *GLUCONOBACTER* FROM OTHER GENERA

See Table BXII.α.10 in the chapter on the family *Acetobacteraceae* for differences of *Gluconobacter* from the other genera of acetic acid bacteria. The medium of Carr and Passmore (1979) provides rapid differentiation of *Gluconobacter* from *Acetobacter*. The genus *Frateruia* (*Frateruia* groups in the *Gammaproteobacteria*) most closely resembles the polarly-flagellated *Gluconobacter* but, in contrast to *Gluconobacter*, is able to oxidize lactate to CO₂/H₂O and does not produce 5-ketogluconic acid. Further information for differentiation of *Gluconobacter* from related genera is given under the section of the family *Acetobacteraceae*.

TAXONOMIC COMMENTS

Within the genus *Gluconobacter*, the species *G. oxydans*, *G. frateruia*, *G. cerinus*, and *G. asaii* were genetically characterized based on 16S rRNA gene sequences (Sievers et al., 1995a) and DNA–DNA hybridization data (Micales et al., 1985; Tanaka et al., 1999). *G. asaii*, *G. frateruia*, and *G. cerinus* showed lower mol% G + C contents of DNA than *G. oxydans*. Phylogenetic trees reflecting the distant and close relationships of *Gluconobacter* based on the results of maximum likelihood analyses are shown in Figs. BXII.α.16 and BXII.α.17 in *Acetobacteraceae*. The overall 16S rRNA gene sequence similarities within the members of the genus *Gluconobacter* are 97.8% to 98.8%, corresponding to 14 to 32 base differences. All four species of *Gluconobacter* form a coherent, closely related cluster that is separated from the other genera of the family *Acetobacteraceae* (Sievers et al., 1994a, 1995a). The ubiquinone composition in *G. oxydans* ATCC 19357 is 3% of Q-9 and 97% of Q-10, in *G. cerinus* IFO 3267, and in *G. asaii* IFO 3276: 4% of Q-9 and 96% of Q-10, respectively, and in *G. frateruia* IFO 3264: 7% Q-9 and 93% Q-10 (Tanaka et al., 1999).

Based on DNA–DNA hybridization data, *G. cerinus* IFO 3262, 3263, 3269 and *G. asaii* IFO 3265 were identified as *G. frateruia* (Tanaka et al., 1999). *G. asaii* IFO 3265 is phenotypically similar to *G. frateruia* (Mason and Claus, 1989). Due to the high values of DNA relatedness (84–96%) between the type strains of *Gluconobacter cerinus* and *Gluconobacter asaii*, *G. asaii* is considered a junior subjective synonym of *G. cerinus* (Yamada et al., 1999; Katsura et al., 2002).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *GLUCONOBACTER*

G. oxydans, *G. frateruia*, *G. cerinus*, and *G. asaii* are differentiated phenotypically by the requirement for nicotinic acid, growth on D-ribitol and L-arabitol (Mason and Claus, 1989), and the formation of acid from D-arabitol, D-ribitol, and L-arabitol (Tanaka

et al., 1999) (Table BXII.α.25). Three transfers into nicotinate-deficient media are necessary to demonstrate nicotinate dependence (Mason and Claus, 1989). The nicotinate-deficient medium is composed of 1% vitamin-free Casamino acids (Difco) and 1% mannitol adjusted to pH 6.0, plus a vitamin solution to achieve the final concentrations of 0.00015% pyridoxal hydrochloride, 0.00015% riboflavin, 0.0001% biotin, 0.0001% thiamine, 0.0001% pantothenic acid, and 0.0001% para-aminobenzoic acid.

Differential characteristics of the species of the genus *Gluconobacter* are given in Table BXII.α.25.

1. GYC medium (g per liter of distilled water): D-glucose, 100.0; yeast extract, 5.0; peptone 3.0; CaCO₃, 12.0; agar, 12.0.

2. MYP medium (g per liter of distilled water): D-mannitol, 25.0; yeast extract, 5.0; peptone, 3.0; and agar, 12.0.

3. DSY medium (g per liter of distilled water): D-sorbitol, 50.0; yeast extract, 5.0; peptone 3.0; and agar, 12.0.

TABLE BXII.α.25. Differentiation of *Gluconobacter* species^a

Characteristic	<i>G. oxydans</i>	<i>G. asaii</i>	<i>G. cerinus</i>	<i>G. frateurii</i>
Growth without nicotinate ^b	—	+	+	+
Growth on D-ribitol ^b	—	—	+ ^c	+
Growth on L-arabitol ^b	—	—	+ ^c	+
Acid formation from: ^d				
D-Arabitol	—	+	+	+
D-Ribitol	—	—	—	+
L-Arabitol	—	—	—	+

^aSymbols: see standard definitions.^bData from Mason and Claus (1989) and Yamada and Akita (1984a).^cNegative strains are present.^dData from Tanaka et al. (1999). The medium (8 ml) for testing of acid formation contained (g per liter of distilled water): pentitol (D-arabitol, D-ribitol, or L-arabitol), 5.0; peptone, 5.0; yeast extract, 3.0; bromocresol purple, 0.02. Acid formation was judged by change of color from purple to yellow (Tanaka et al., 1999). Formation of acid by the use of this medium was determined by the authors with the type strains of the *Gluconobacter* species and some isolates.List of species of the genus *Gluconobacter*1. *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961, 304^{AL} emend. Mason and Claus 1989, 181.*ox'y. dans*. M.L. part. adj. *oxydans* acid-giving, oxidizing.

All strains produce 2-ketogluconic acid, 5-ketogluconic acid, and 2,5-diketogluconic acid from D-glucose and dihydroxyacetone from glycerol. *G. oxydans* strains grow to an OD₆₂₀ nm of less than 1.0 after 24 h of incubation and three passages (24 h of incubation each) in media lacking nicotinate. All strains grow to an OD₆₂₀ nm of 0.5 or less in medium containing ribitol or arabitol as the primary carbon source (Mason and Claus, 1989). All strains produce acid from ethanol, *n*-propanol, D-glucose, D-arabinose, D-fructose, D-ribose, D-mannose, D-sorbitol, and D-mannitol, and tolerate up to 10% D-glucose. Carbon sources for growth are D-mannitol, glycerol, or D-sorbitol. Batch cultures of *G. oxydans* have shown that glucose is the preferred substrate of this species rather than lactate (Poget et al., 1994). The type strain of *G. oxydans*, ATCC 19357, does not form acid from D-arabitol, ribitol, L-arabitol, and sucrose (Tanaka et al., 1999).

G. oxydans is not further divided into subspecies, based on cluster analysis of protein profiles from *Gluconobacter* strains (Gosselé et al., 1983a).

The genome sizes of four *G. oxydans* strains were estimated to be between 2240 and 3787 kb by pulsed-field gel electrophoresis upon *Xba*I digestion of their genomes (Verma et al., 1997).

The mol% G + C of the DNA is: 56–64 (*T_m*).

Type strain: ATCC 19357, IFO 14819, DSM 7145.

GenBank accession number (16S rRNA): X73820.

2. *Gluconobacter asaii* Mason and Claus 1989, 183^{VP}

a.sa'i.i. M.L. n. *asaii* of Toshinobu Asai, Japanese microbiologist, who described the genus *Gluconobacter*.

All strains produce acid from ethanol, D-glucose, D-xylose, maltose, and tolerate up to 10% D-glucose. *G. asaii* strains grow to an OD₆₂₀ nm of 1.0 or more after 24 h of incubation and three passages (24 h of incubation each) in media lacking nicotinate. All strains fail to grow beyond an OD₆₂₀ of 0.5 after 24 h when either ribitol or arabitol is used as the primary carbon source. *G. asaii* strain IFO 3297a is characterized by its unique ability to cause alkalization of polyol media during growth on mannitol, ribitol, or arabitol. The polyol medium is composed of (g

per liter of distilled water): vitamin-free Casamino acids (Difco), 3.0; yeast extract, 5.0; and polyol (mannitol, ribitol, or arabitol), 5.0 (Mason and Claus, 1989). The type strain for *G. asaii* (IFO 3276) does not form acid from ribitol, L-arabitol, and sucrose. Since *G. asaii* IFO 3276 showed species-level similarity of 88% by DNA–DNA hybridization with *G. cerinus* IFO 3267 (Tanaka et al., 1999); *G. asaii* IFO 3276 does not represent the species *Gluconobacter asaii* as type strain.

The mol% G + C of the DNA is: 52–55 (*T_m*).

Type strain: ATCC 49206, IFO 3276, DSM 7148.

GenBank accession number (16S rRNA): AB063287.

3. *Gluconobacter cerinus* Yamada and Akita 1984b, 503^{VP} (Effective publication: Yamada and Akita 1984a, 124.)

ce.n'i' nus. L. adj. *cerinus* wax-colored.

Cells are polarly flagellated when motile. Colonies are glossy and smooth. Produces 2-ketogluconate and 5-ketogluconate from glucose. 2,5-Diketogluconate is not produced from D-glucose. Produces dihydroxyacetone from glycerol. Growth occurs on ribitol, xylitol, and L-arabitol. Requires pantothenic acid but not nicotinate for growth. All strains produce acid from ethanol, D-glucose, and D-fructose.

By comparison of electrophoretic patterns from enzymes, the differentiation of *G. cerinus* and *G. oxydans* strains as two separate groups was shown (Yamada and Akita, 1984a).

G. cerinus and *G. frateurii* are phenotypically similar in respect to growth on ribitol and L-arabitol (Yamada and Akita, 1984a; Mason and Claus, 1989). The type strain for *G. cerinus* IFO 3267, in contrast to *G. frateurii* IFO 3264, does not form acid from ribitol and L-arabitol. Weak formation of acid from sucrose is observed by *G. cerinus* IFO 3267 (Tanaka et al., 1999). The type strains of *G. cerinus* and *G. frateurii* have a DNA–DNA similarity value of 23% (Micales et al., 1985) and a 16S rRNA gene sequence similarity value of 98.8%, corresponding to 15 nucleotide differences (Sievers et al., 1995a). The two colony types (glossy and smooth) of the type strain *G. cerinus* IFO 3267 have identical 16S rRNA gene sequences.

The mol% G + C of the DNA is: 54–58 (*T_m*).

Type strain: ATCC 19441, IFO 3267, DSM 9533.

GenBank accession number (16S rRNA): AB063286.

4. **Gluconobacter frateurii** Mason and Claus 1989, 182^{VP}
fra.teur'i.i. M.L. n. *frateurii* of Joseph Frateur (1903–1974),
 Belgian microbiologist, who is well known for his study of
 acetic acid bacteria.

All strains produce acid from ethanol, glucose, xylose, maltose, sorbitol, mannitol, lactose, ribitol, and *myo*-inositol, and tolerate up to 15% D-glucose. *G. frateurii* strains grow to an OD₆₂₀ nm of 1.0 or more after 24 h of incubation and three passages (24 h of incubation each) in media

lacking nicotinate (except strain IFO 3272). All strains grow to an OD₆₂₀ nm of 1.0 or more after 24 h when either ribitol or arabinol is used as the primary carbon source (Mason and Claus, 1989). The type strain *G. frateurii* IFO 3264 forms acid from D-arabinol, ribitol, L-arabinol, and sucrose (Tanaka et al., 1999).

The mol% G + C of the DNA is: 53–55 (*T_m*).

Type strain: ATCC 49207, IFO 3264, DSM 7146.

GenBank accession number (16S rRNA): X82290.

Genus X. **Paracraurococcus** Saitoh, Suzuki and Nishimura 1998, 1045^{VP}

CHRISTOPHER RATHGEBER AND VLADIMIR V. YURKOV

Pa.ra.cra.u.ro.coc'cus. Gr. prep. *para* like, along side of; Gr. adj. *crauros* fragile; Gr. n. *coccus* berry; M.L. masc. n. *Paracraurococcus coccus* like *Craurococcus*.

Gram-negative, nonmotile cocci, 0.8–1.5 µm in diameter. Cell division occurs by binary fission. Cells grow heterotrophically under aerobic conditions giving rise to red colonies on solid media. **Obligately aerobic, containing bacteriochlorophyll (bchl) *a* organized into reaction center and light-harvesting complexes, as well as carotenoid pigments. No growth occurs anaerobically under illuminated conditions.** Facultatively photoheterotrophic. The habitat is soil.

Type species: **Paracraurococcus ruber** Saitoh, Suzuki and Nishimura 1998, 1046.

FURTHER DESCRIPTIVE INFORMATION

P. ruber is currently the only known representative of the genus *Paracraurococcus*. Phylogenetically this species belongs to the *Alphaproteobacteria*, most closely related to the genera *Craurococcus*, *Roseococcus*, *Rhodospila*, and *Acidiphilium*.

P. ruber forms small (<1mm diameter), irregular, red-to-dark-red colonies on agar media (Fig. BXII.α.28A). Circular, convex, and smooth colonies may appear; these colonies have physiological properties equivalent to strains forming irregular colonies.

Cells of *P. ruber* grown in NPG (Saitoh and Nishimura, 1996) medium adjusted to pH 6.8 appear as cocci. The cells range in size from 0.8 to 1.5 µm in diameter, appear singly or in pairs, and often form aggregates. Cells are nonmotile, spore production has not been observed. *P. ruber* accumulates poly-β-hydroxybutyrate granules when grown under regular culture conditions. Cell division is by way of binary fission (Fig. BXII.α.28B).

Divalent cations appear to be required to maintain proper cell structure in liquid media. Cells lyse when suspended in aqueous solution, however, lysis can be prevented by the addition of divalent cations such as Ca²⁺ or Mg²⁺. Additions of NaCl, KCl, and sucrose are not sufficient to prevent lysis. The addition of divalent cations to culture media is not required for growth, but has been shown to promote growth in all strains. When cells are grown on solid media, addition of divalent cations is not required and does not affect growth rate.

Intact cells of *P. ruber* show absorption spectrum peaks at 802 nm and 856 nm, corresponding to Bchl *a* organized into reaction center and light-harvesting complexes, respectively. Crude pigment extracts in methanol show absorption spectrum peaks confirming the presence of carotenoid pigments (362 and 492nm) and Bchl *a* (770 nm). Bchl *a* levels have been estimated at 0.3 nmol/g dry cell weight, with slightly higher levels found in strains forming circular, smooth colonies. Cells grown under illuminated conditions have been shown to contain lower levels of Bchl *a*, as compared to cells grown in the dark, indicating that illumination

suppresses the synthesis of Bchl *a* in these strains (Saitoh and Nishimura, 1996). Strong inhibition of Bchl *a* synthesis by light has been detected in many aerobic phototrophic bacteria (Yurkov and Beatty, 1998a). Absorption difference spectra (illuminated minus unilluminated) of membrane fractions indicate a photochemically active photosynthetic apparatus, similar to those

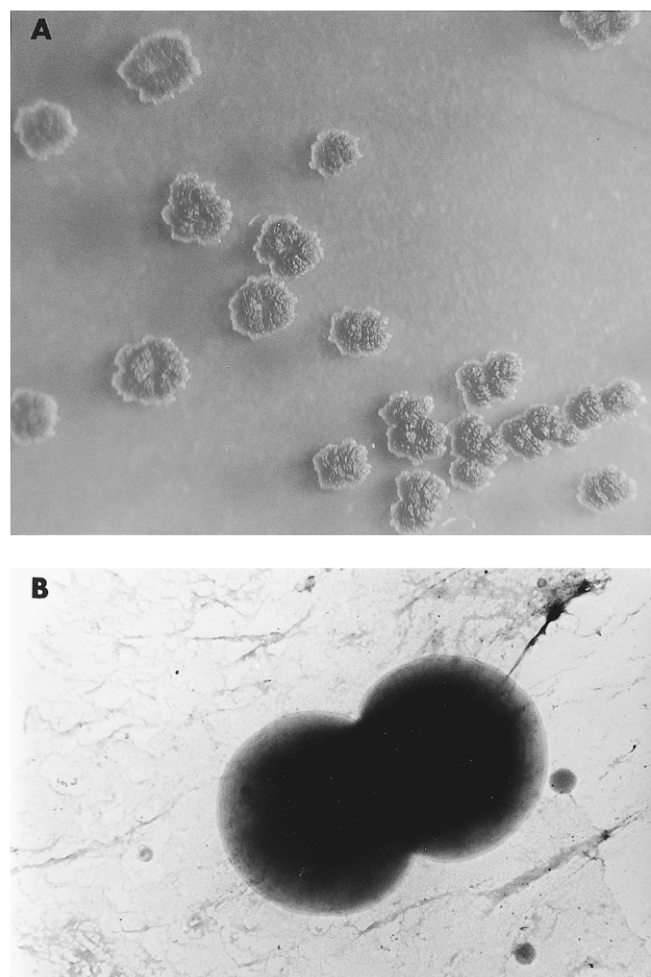


FIGURE BXII.α.28. *Paracraurococcus ruber*. (A) Photograph showing red, irregular colonies grown on agar plates. (B) Electron microscopic photograph showing division by binary fission. (Courtesy of Y. Nishimura.)

found in other aerobic, as well as typical anaerobic phototrophic bacteria (Saitoh and Nishimura, 1996). *P. ruber* cells contain large amounts of carotenoid acids and smaller amounts of spirilloxanthin.

The cellular fatty acid composition of *P. ruber* consists of octadecanoic acid as well as smaller amounts of octadecadienoic acid, 2-hydroxyoctadecenoic acid, 3-hydroxytetradecanoic acid, and 3-hydroxyoctadecanoic acid. Ubiquinone is the only quinone detected. The major quinone is Q-10 and the quinones Q-7, Q-8, and Q-9 occur in smaller quantities. The cellular lipid profile consists of phospholipids of phosphatidylethanolamine, bisphosphatidylglycerol, and phosphatidylglycerol (Saitoh and Nishimura, 1996).

P. ruber grows heterotrophically under aerobic conditions, and does not grow anaerobically under either illuminated or unilluminated conditions. However light intensities of about 2000 lux stimulate growth under aerobic conditions. Organic substrates utilized are D-xylose, fumarate, L-glutamate, glycerol, D-fructose, D-galactose, D-glucose, D-arabinose, L-rhamnose, D-ribose, D-fucose, DL-lactate, malonate, pyruvate, gluconate, and L-malate. Acids are produced oxidatively from sugars. Hydrolyzes Tween 20, 40, and 60. Tween 80 and gelatin are not hydrolyzed.

NaCl is not required for growth and cells do not grow above 0.4% NaCl. Susceptible to chloramphenicol, streptomycin, and tetracycline, and resistant to penicillin and polymyxin B.

Mesophilic, with growth occurring between 16°C and 42°C, with an optimum growth temperature between 30°C and 34°C. The pH range is between 5.5 and 8.1, with optimum growth occurring at pH 6.6–6.8. Nicotinic acid and pantothenate are required as growth factors.

ENRICHMENT AND ISOLATION PROCEDURES

Two strains of *P. ruber* were isolated from soil samples taken from a playground in Yamasaki Noila, of Osakajo Park in Chuoh Ward, Osaka, on a 100-fold dilution of nutrient agar medium supplemented with 1.5% agar.

MAINTENANCE PROCEDURES

Strains can be maintained on a NPG medium (Saitoh and Nishimura, 1996) adjusted to pH 6.8. Long-term storage procedures have not been described.

List of species of the genus *Paracraurococcus*

1. ***Paracraurococcus ruber*** Saitoh, Suzuki and Nishimura 1998, 1046^{VP}

ru.ber. L. adj. *ruber* red-colored, red bacterium.

Gram-negative cocci, 0.8–1.5 µm in diameter, nonmotile, divide by means of binary fission. Cells contain Bchl *a* organized into reaction center and light-harvesting complexes, giving rise to absorption spectrum peaks at 802 and 856 nm, respectively, as well as spirilloxanthin and carotenoid acids. Facultative photoheterotrophs, incapable of anaerobic growth even under illuminated conditions. Aerobic growth is stimulated by light intensities of 2000 lux. Best growth substrates include D-xylose, fumarate and L-glutamate. Glycerol, D-fructose, D-galactose, D-glucose, D-arabinose, L-rhamnose, D-ribose, D-fucose, DL-lactate, malonate, pyruvate, gluconate, and L-malate also serve as the sole carbon source for heterotrophic growth. Incapable of growth on D-mannitol, sorbitol, methanol, ethanol, 1,2-propanediol, kerosene, acetate, butyrate, citrate, formate, glycolate, propionate, succinate, phthalate, *p*-hydroxybenzoate, and

DIFFERENTIATION OF THE GENUS *PARACRAUROCOCCUS* FROM OTHER GENERA

Paracraurococcus can be differentiated from other genera based on several characteristics. The presence of Bchl *a* organized into reaction center and light-harvesting complexes can be shown by absorption spectrum peaks at 802 and 856 nm, respectively. Light-harvesting complex I, absorbing at 856 nm, is very rarely found among photosynthetic bacteria. The only known species that contains similar light-harvesting complexes is *Roseococcus thiosulfatophilus* (Yurkov and Beatty, 1998a). The presence of Bchl *a* and the inability to grow under illuminated, anaerobic conditions distinguishes *Paracraurococcus* from members of the purple non-sulfur bacteria and places it among the obligately aerobic phototrophic bacteria.

Paracraurococcus can be differentiated from other genera of aerobic phototrophs based on color, red-to-dark-red colonies, as well as its unusual requirement for divalent cations to maintain cell structure in suspension.

On a phylogenetic tree, *Paracraurococcus* forms a separate line of descent with the closely related genus *Craurococcus*. However, *Paracraurococcus* can be distinguished from *Craurococcus* based on differences in absorption spectrum characteristics. *Paracraurococcus* shows absorption spectrum peaks at 802 and 856 nm, whereas *Craurococcus* shows absorption spectrum peaks at 800 and 872 nm, indicating a difference in the structural organization of the light-harvesting complex I between the two genera (see Table BXII.α.23 in Genus *Craurococcus*).

TAXONOMIC COMMENTS

16S rRNA gene sequence analysis shows that *Paracraurococcus* forms a separate line of descent, along with *Craurococcus*, within the *Alphaproteobacteria*. This line falls within a cluster formed by the genera *Roseococcus*, *Rhodospila*, and *Acidiphilium* (Saitoh et al., 1998).

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.
- Yurkov, V.V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

benzoate. Hydrolyze Tween 20, 40, and 60, but do not hydrolyze Tween 80. Produce catalase, oxidase, and urease. Reduce nitrate to nitrite. Denitrification not observed. Slight hydrolysis of starch. Do not hydrolyze esculin or gelatin. Do not produce phosphatase, deoxyribonuclease, or indole, but do produce H₂S. Voges–Proskauer test and methyl-red test are negative. Accumulate poly-β-hydroxybutyrate granules.

Optimal growth occurs at pH 6.6–6.8 and 30–34°C. Incapable of growth in NaCl concentrations higher than 0.4%. Require divalent cations to maintain cell structure in suspension. Sensitive to chloramphenicol, streptomycin, and tetracycline. Resistant to penicillin and polymyxin B. Nicotinic acid and pantothenate are required as growth factors. Habitat: soil from a playground located in Yamazaki, Noda, of Osakajo Park in Chuoh Ward, Osaka, Japan.

The mol% G + C of the DNA is: 70.3–71.0 (HPLC).

Type strain: NS89, CIP 105708, JCM 9931.

GenBank accession number (16S rRNA): D85827.

Genus XI. Rhodopila Imhoff, Trüper and Pfennig 1984, 341^{VP}

MICHAEL T. MADIGAN AND JOHANNES F. IMHOFF

Rho.do.pi la. Gr. n. *rhodon* the rose; M.L. fem. n. *pila* a ball or sphere; M.L. fem. n. *Rhodopila* red sphere.

Cells are spherical to ovoid, motile by means of polar flagella, and divide by binary fission (Fig. BXII.α.29). **Gram negative, belonging to the Alphaproteobacteria.** Internal photosynthetic membranes are of the vesicular type. **Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids.** Major fatty acids are C_{18:1} (~75%) and C_{16:0}. Ubiquinones, menaquinones, and rhodoquinones with 9 and 10 isoprene units are present.

Growth occurs preferably photoheterotrophically under anoxic conditions in the light. Cells may be sensitive to oxygen but grow under microoxic conditions in the dark. Growth factors are required. Acidophilic freshwater bacteria that live in warm sulfur springs.

The mol% G + C of the DNA is: 66.3.

Type species: *Rhodopila globiformis* (Pfennig 1974) Imhoff, Trüper and Pfennig 1984, 341 (*Rhodopseudomonas globiformis* Pfennig 1974, 205.)

FURTHER DESCRIPTIVE INFORMATION

Rhodopila globiformis is the only known species of this genus and is characterized by a number of peculiar physiological properties. It is an acidophilic bacterium, capable of growth at the lowest pH known for phototrophic purple nonsulfur bacteria (PNSB) (Pfennig, 1969a, 1974). Different pH optima for *Rhodopila globiformis* have been noted during growth with mannitol (pH 4.8–5.0) and fumarate (pH 5.6) as carbon sources (Pfennig, 1974). Defining characteristics are given in Table BXII.α.26; carbon sources and electron donors used are given in Table BXII.α.27. The genus is compared to other phototrophic members of the *Rhodospirillales* in Tables 1 and 2 of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria" (Volume 2, Part A, pp. 121–123). The relationships of these bacteria based on 16S rDNA analysis are shown in Fig. 1 (p. 124) of that chapter.

Rhodopila globiformis is a photoheterotroph, unable to use most of the carbon substrates used by other phototrophic purple non-sulfur bacteria. Ammonia is assimilated via the glutamine synthetase/glutamate synthase reaction (NADH-dependent). Glutamate dehydrogenase is not present, but low activities of an alanine dehydrogenase were found (Madigan and Cox, 1982).

Growth with sulfate as sole sulfur source is possible at low concentrations, but at more than 1 mM sulfate, growth is inhibited. The inhibitory effects of higher sulfate concentrations are thought to be due to misregulation of the enzymes involved (Hensel and Trüper, 1976; Imhoff et al., 1981) and can be overcome by supplementation with a suitable reduced sulfur source. All enzymes necessary for sulfate assimilation are present. Sulfate is reduced via adenosine-5'-phosphosulfate (Imhoff, 1982). Good sulfur sources are cysteine and thiosulfate. Some of the latter is assimilated, but most of it is oxidized to tetrathionate (Then and Trüper, 1981). Sulfite and sulfide are growth-inhibitory even at low concentrations.

Rhodopila globiformis has unique aliphatic ketocarotenoids as major components (Schmidt and Liaaen-Jensen, 1973; Schmidt, 1978) and small amounts of asymmetrical gamma-carotene and neurosporene (S. Takaichi, personal communication). *Rhodopila globiformis* also contains unusual polar lipids (Imhoff et al., 1982), a "small type" cytochrome *c*₂ (Dickerson, 1980b), and is among the few species of the PNSB that have readily detectable amounts of a high potential iron-sulfur protein (HiPIP).

The type strain of *Rhodopila globiformis* was isolated from a weak red layer present in an acidic sulfur spring along the Gibbon River, Yellowstone National Park (USA). There are several warm springs in this area which are located on the east side of the Grand Loop Road in Yellowstone, about 100 m northeast of Beryl Spring. The springs are warm (~40°C), contain large amounts of S⁰ (presumably from the oxidation of sulfide), and can be

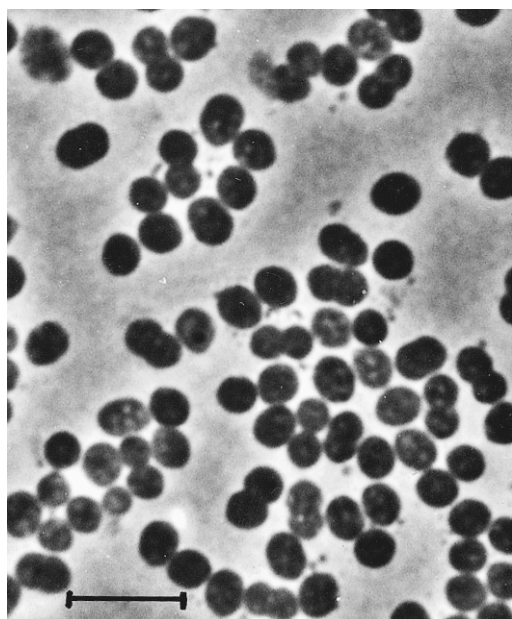


FIGURE BXII.α.29. Phase-contrast photomicrograph of cells of *Rhodopila globiformis* strain DSM 161^T. Bar = 5 μm. (Courtesy of N. Pfennig.)

TABLE BXII.α.26. Characteristics of *Rhodopila globiformis*^a

Characteristic	<i>Rhodopila globiformis</i>
Cell diameter (μm)	1.6–1.8
Internal membrane system	Vesicles
Motility	+
Color	Purple-red
Bacteriochlorophyll	<i>a</i>
Growth factors	Biotin, <i>p</i> -aminobenzoic acid
Aerobic growth	(+)
Oxidation of sulfide	–
Salt requirement	None
Optimal temperature (°C)	30–35
Optimal pH	4.8–5.0
Habitat	Fresh water
Mol% G + C of the DNA ^c	66.3
Cytochrome <i>c</i> size	Small
Major quinones	Q-9/10, MK-9/10, RQ-9/10
Major fatty acids:	
C _{14:0}	5.8
C _{16:0}	9.3
C _{16:1}	4.7
C _{18:0}	1
C _{18:1}	74.4

^aSymbols: +, positive in most strains; –, negative in most strains; (+), weak growth or microaerobic growth only; nd, not determined; Q-9/10, ubiquinones 9 and 10; MK-9/10, menaquinones 9 and 10; RQ-9/10, rhodoquinones 9 and 10.

TABLE BXII.α.27. Carbon sources and electron donors used by *Rhodopila globiformis*^a

Source/donor	<i>Rhodopila globiformis</i>
<i>Carbon sources:</i>	
Acetate	—
Arginine	—
Aspartate	—
Benzoate	—
Butyrate	—
Caproate	—
Caprylate	—
Citrate	—
Ethanol	+
Formate	—
Fructose	+
Fumarate	+
Glucose	+
Glutamate	—
Glycerol	—
Glycolate	—
Lactate	—
Malate	+
Mannitol	+
Methanol	—
Pelargonate	—
Propionate	—
Pyruvate	+
Succinate	+
Tartrate	+
Valerate	—
<i>Electron donors:</i>	
Hydrogen	nd
Sulfide	nd
Sulfur	—
Thiosulfate	—

^aSymbols: Symbols: +, positive in most strains; —, negative in most strains; +/—, variable in different strains; nd, not determined.

very acidic. Measurements in a series of such springs showed pH values as low as 2.7, with most of them in the range of pH 3–4 (M.T. Madigan, unpublished results). Other locations in Yellowstone where *Rhodopila globiformis* has been observed include warm sulfur springs on the periphery of Nymph Lake; an isolate of *R. globiformis* (strain NL) that matches the type strain in most phenotypic properties was isolated from such a spring (M.T. Madigan, unpublished results). *R. globiformis* coexists in these springs with the extremely acidophilic red alga *Cyanidium caldarium* (Brock, 1978), which supports evidence of the true acidophilic nature of *R. globiformis*.

ENRICHMENT AND ISOLATION PROCEDURES

If samples from suitable natural habitats are available, *Rhodopila globiformis* can be selectively enriched in media with gluconate (or ethanol) as carbon source, thiosulfate as sulfur source, and ammonia or N₂ as nitrogen source at acidic pH (4.8–5.0). Isolation can be achieved by standard procedures for phototrophic purple bacteria under strictly anoxic conditions (Imhoff, 1988; Imhoff and Trüper, 1992). Agar shake cultures established using samples of suspected blooms of *R. globiformis* yield cherry-red colonies within one week at 35°C and 1000 lux incandescent illumination. A suitable medium for *R. globiformis* (after Pfennig, 1974) is given in the footnote below.¹

1. The medium contains (per liter): KH₂PO₄, 0.5 g; NH₄Cl, 0.4 g; MgSO₄·7H₂O, 0.4 g; NaCl, 0.4 g; CaCl₂·2H₂O, 0.05 g; Na₂S₂O₃·5H₂O, 0.2 g; ferrous citrate, 0.005 g; mannitol, 1.5 g; gluconate, 0.5 g; biotin, 100 µg; *p*-aminobenzoic acid, 200 µg; and trace element solution 6, 10 ml. The initial pH is adjusted to 5.0.

MAINTENANCE PROCEDURES

Cultures of *R. globiformis* are well preserved in liquid nitrogen or at –80°C in a mechanical freezer. Mid-exponential phase cell suspensions should be mixed with glycerol or DMSO to yield final concentrations of 10% and 5%, respectively, and kept at 0°C for 15 min and then frozen immediately.

DIFFERENTIATION OF THE GENUS *RHODOPILA* FROM OTHER GENERA

The genus *Rhodopila* is represented by a single species. *Rhodopila globiformis* differs from all other anoxygenic phototrophic *Alphaproteobacteria* in its outstanding physiological and chemotaxonomic properties and phylogenetic position based on 16S rDNA sequence analysis. First and foremost, acidophily (Pfennig, 1974) and unique carotenoid composition (Schmidt, 1978) characterize *Rhodopila globiformis*. Furthermore, cytochrome *c*₂ structure (Dickerson, 1980b), quinone and polar lipid composition (Imhoff and Bias-Imhoff, 1995), sulfate assimilation via adenosine-5'-phosphosulfate (Imhoff, 1982), presence of a high potential iron-sulfur protein (HiPIP), and 16S rDNA sequence are characteristics that differentiate *Rhodopila* from other phototrophic *Alphaproteobacteria*. Comparative 16S rDNA sequence analysis of *R. globiformis* shows a clear relationship to both phototrophic and chemotrophic species. A particularly close relationship exists to the acidophilic chemotrophic bacteria including *Acidiphilium* species (Fig. 1 p. 124 in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A; see also Fig. 1 p. 136 in the introductory chapter "Aerobic Bacteriochlorophyll-Containing Bacteria", Volume 2, Part A). This suggests that the acidophilic lifestyle of *R. globiformis* is a fundamental physiological property rooted in its phylogenetic ties to other acidophilic bacteria. The close relationship to *Acidiphilium* species is of particular interest, because these bacteria contain an extraordinary bacteriochlorophyll *a* with Zn instead of Mg as the chelated metal ion (Wakao et al., 1993, 1996; see Genus *Acidiphilium*).

TAXONOMIC COMMENTS

Several outstanding characteristics of *Rhodopila globiformis* warrant its treatment as a separate genus (Imhoff et al., 1984). This species was originally described as *Rhodopseudomonas globiformis* (Pfennig, 1974) and later reclassified based on unique physiological, chemotaxonomic, and phylogenetic characteristics (Imhoff et al., 1984).

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List of species of the genus *Rhodopila*

1. ***Rhodopila globiformis*** (Pfennig 1974) Imhoff, Trüper and Pfennig 1984, 341^{VP} (*Rhodopseudomonas globiformis* Pfennig 1974, 205.)

glo.bi.for' mis. L. n. *globus* sphere; L. n. *forma* shape; M.L. n. *globiformis* of spherical shape.

Cells are spherical to ovoid, diplococcus-shaped before cell division, under optimal growth conditions 1.6–1.8 µm in diameter (depending on the culture conditions diameter ranges from 1.0 to 2.5 µm), and motile by means of polar flagella. Internal photosynthetic membranes are of the vesicular type. Color of cultures grown anaerobically in the light is intensely purple-red; microaerobically grown cells are pink. Absorption spectra of living cells show maxima at 378, 594, 813, and 862 nm and a shoulder at 890 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and as major components unusual aliphatic ketocarotenoids.

Cells grow preferably photoheterotrophically under anoxic conditions in the light, but also chemotrophically under microoxic conditions in the dark. Best growth is obtained with gluconate, mannitol, fructose, or ethanol as

carbon source. Glucose, tartrate, fumarate, succinate, malate, pyruvate, and yeast extract at low concentration are also assimilated. No growth occurs with fatty acids, lactate, citrate, glycerol, mannose, sorbitol, amino acids, and benzoate as carbon source. Besides ammonia, which is the best nitrogen source, N₂, glutamate, glutamine, aspartate, arginine, urea, asparagine, and alanine are used. Peptone and yeast extract are utilized at low concentrations (0.05%) but are growth-inhibitory at higher concentrations. Nitrate is not assimilated. Thiosulfate, cysteine, or low concentrations of sulfate are the best sulfur sources. Sulfate is growth inhibitory at concentrations above 1 mM. Methionine and tetrathionate can also be used, but sulfide and sulfite inhibit growth even at low concentrations. Biotin and *p*-aminobenzoic acid are required as growth factors.

Acidophilic freshwater bacterium with optimal growth at 30–35°C (no growth at and above 40°C) and at pH 4.8–5.0 with mannitol as carbon source (pH range: 4.2–6.5).

The mol% G + C of the DNA is: 66.3 (Bd).

Type strain: Pfennig 7950, ATCC 35887, DSM 161.

GenBank accession number (16S rRNA): D86513.

Genus XII. *Roseococcus* Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 430^{VP}

VLADIMIR V. YURKOV

Ro'se.o.coc' cus. M.L. adj. *roseus* rose, pink; Gr. n. *coccus* sphere or spheroidal shape; M.L. masc. n. *Roseococcus* pink spherical bacterium.

Cells are Gram negative, coccoidal, pink and motile by means of polar flagella. Divide by binary fission. **Bacteriochlorophyll *a* and carotenoid pigments** are present. **Obligately aerobic, chemoorganotrophic** (respiratory metabolism) and **facultatively photoheterotrophic**. **No growth occurs under anaerobic conditions in the light**. May use thiosulfate as an additional energy source. Methanol is not utilized. NaCl not required for growth.

The mol% G + C of the DNA is: 70.4.

Type species: ***Roseococcus thiosulfatophilus*** Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 432.

FURTHER DESCRIPTIVE INFORMATION

The genus *Roseococcus* is phylogenetically related to members of the *Alphaproteobacteria*; this class also includes other aerobic anoxygenic phototrophic bacteria as well as purple nonsulfur photosynthetic and several nonphotosynthetic species. *Roseococcus* is moderately related to *Rhodopila globiformis*, *Thiobacillus acidophilus*, and species of the genus *Acidiphilium* (levels of 16S rDNA sequence homology, >90%) (Yurkov et al., 1994c).

Only one species of the genus is currently isolated and described, *Roseococcus thiosulfatophilus*, represented by pink-red coccoidal cells of 0.9–1.3 × 1.3–1.6 µm in size (Fig. BXII.α.30) and motile by means of a single polar flagellum. The pink-red color

of this species is due mainly to two polar red pigments, C₃₀ carotene-dioate (4,4'-diapocarotene-4,4'-dioate) and the respective diglucosyl ester (di[β-D-glucopyranosyl]-4,4'-diapocarotene-4,4'-dioate). Together they contribute 95% of the total carotenoid content (Yurkov et al., 1993). Such highly polar C₃₀ carotenoid glycosides have never before been observed in other phototrophic bacteria, although the same carotenoid and its diglucosylated form have previously been postulated to exist in *Methylobacterium rhodinum* (formerly *Pseudomonas rhodos*) (Yurkov et al., 1993).

Absorption spectra of the intact cells of *R. thiosulfatophilus* yielded several peaks: at 482, 510, 538, 800, and 859 nm (Yurkov and Gorlenko, 1991). Absorption peaks at 482, 510, and 538 nm are due to the presence of two highly polar red carotenoids described above. Absorption peaks in the infra-red spectrum region indicate the incorporation of bacteriochlorophyll *a* into the reaction center (minor peak at 800 nm) and an unusual type of light-harvesting complex (major peak at 859 nm). Subsequent purification of light-harvesting complexes from these bacteria by detergent treatment of membranes and sucrose density gradient centrifugation revealed the existence of a new *R. thiosulfatophilus* light-harvesting complex I with an absorption peak at 856 nm. The reaction center of *R. thiosulfatophilus* possesses a tightly bound cytochrome *c* (of 44 kDa) that serves as the immediate

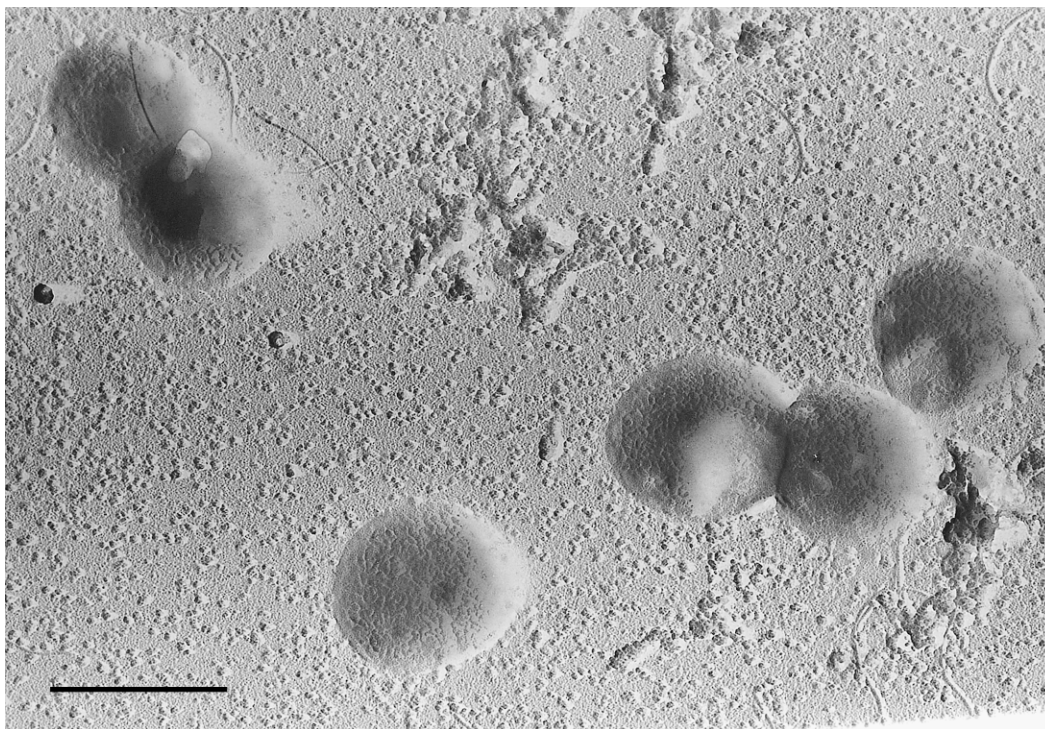


FIGURE BXII.α.30. Coccoid cells of *Roseococcus thiosulfatophilus*. Scanning electron micrograph of carbon shadowed cells. Bar = 1.0 μm. (Courtesy of V. Yurkov.)

electron donor to the photooxidized reaction center. The photosynthetic apparatus of *R. thiosulfatophilus* was shown to be functional in terms of a cyclic electron transfer under aerobic conditions. It is not functional anaerobically (Yurkov et al., 1995, 1998a).

Most of the *R. thiosulfatophilus* carotenoids are not bound to the bacteriochlorophyll-protein complexes of the photosynthetic apparatus but to the envelope fraction (cytoplasmic membrane and cell wall) (Yurkov et al., 1993). The excess of carotenoids in the membranes does not contribute to the light-harvesting function of the light-harvesting complexes (Yurkov et al., 1994a).

R. thiosulfatophilus is highly resistant to the toxic heavy metal oxide tellurite (Yurkov et al., 1996). However, the resistance of *R. thiosulfatophilus* to tellurite is not always correlated to its reduction to tellurium. High-level resistance without tellurite reduction was observed for *R. thiosulfatophilus* grown with L-glutamine, succinate, malate, tartrate, or acetate as the organic carbon sources. Tellurite reduction with intracellular deposition of small tellurium crystals in *R. thiosulfatophilus* was shown in rich organic medium (Fig. BXII.α.31). These results are similar to that observed for *Erythromicrobium ezovicum*, implying that tellurite reduction is not essential to confer tellurite resistance and some other important mechanisms could play a role in the resistance character (Yurkov et al., 1996).

ENRICHMENT AND ISOLATION PROCEDURES

R. thiosulfatophilus was isolated in pure culture from a cyanobacterial mat developed in an alkaline sulfide high temperature spring situated in the Bol'shaya River valley. Isolation procedures are similar to that described for the genus *Erythromicrobium* (see the chapter on the *Erythromicrobium*).

MAINTENANCE PROCEDURES

Maintenance of *R. thiosulfatophilus* in liquid culture, on agar plates and long-term preservation are the same as described for the genus *Erythromicrobium*.

DIFFERENTIATION OF THE GENUS *ROSEOCOCCUS* FROM OTHER GENERA

The genus *Roseococcus* has many specific features that differentiate this genus from other genera of aerobic anoxygenic phototrophic bacteria: very high mol% G + C DNA content (70.4) and low DNA homology determined by DNA-DNA hybridization, morphological peculiarities (cell shape and color), carotenoid composition, photosynthetic apparatus components, ability to oxidize thiosulfate to sulfate, and resist high concentrations of toxic tellurite in a special manner (See above).

Roseococcus thiosulfatophilus shares high 16S rDNA sequence similarity with the purple nonsulfur bacterium *Rhodospira globiformis*, which is the closest phylogenetic relative (Yurkov et al., 1994c). Both *Roseococcus thiosulfatophilus* and *R. globiformis* contain bacteriochlorophyll *a* and are able to oxidize thiosulfate. Both these microorganisms also form pink colonies and their mol% G + C contents are high (66.3 and 70.4). There are, however, considerable differences in the growth conditions which they prefer; *Rhodospira globiformis* prefers to grow anaerobically under photoheterotrophic conditions, while *Roseococcus thiosulfatophilus* is obligately aerobic. The antenna systems of these two organisms are also different. *Rhodospira globiformis* seems to have two light-harvesting complexes (with absorption peaks at 813, 862, and 890 nm), whereas *Roseococcus thiosulfatophilus* has only one (with an absorption peak at 856 nm).

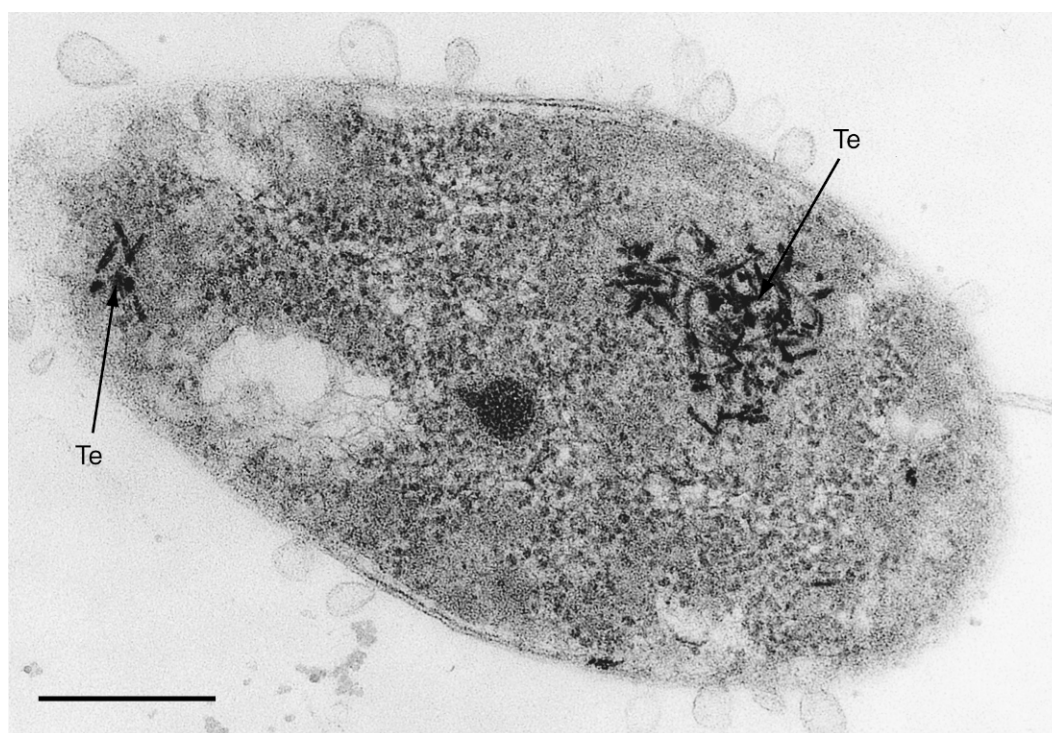


FIGURE XII.31. *Roseococcus thiosulfatophilus*, electron microscopy of ultrathin sections. Intracellular localization of tellurium (Te; indicated by arrows) as a product of tellurite reduction. Bar = 0.25 μm . (Courtesy of V. Yurkov.)

TAXONOMIC COMMENTS

The genus *Roseococcus* was created by Yurkov and Gorlenko in 1991 for bacterial strains isolated from a sulfide high temperature spring, based on their morphological, physiological, and DNA-DNA hybridization analyses (Yurkov and Gorlenko, 1991). A valid description of this genus was supplemented by additional results on the photosynthetic apparatus organization, carotenoid composition, and phylogeny (Yurkov et al., 1994c).

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List of species of the genus *Roseococcus*

1. ***Roseococcus thiosulfatophilus*** Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drets 1994c, 432^{VP}
thi' o.sul.fa.to' phi.lus. M.L. adj. *thiosulfatophilus* thiosulfate liking.

Gram-negative, pink cocci that are $0.9\text{--}1.3 \times 1.3\text{--}1.6 \mu\text{m}$ and are motile by means of single polar flagellum. Cells contain bacteriochlorophyll *a* and carotenoids. Membranes isolated from cells grown semiaerobically in the dark have absorption maxima at 478, 501, and 505 nm (carotenoids) and at 800 and 855 nm (bacteriochlorophyll). The major carotenoid is C₃₀ carotene-dioate. Aerobic, chemoorganotrophic, and facultatively photoheterotrophic.

Growth occurs on yeast extract and when succinate, acetate, pyruvate, citrate, lactate, malate, or glutamate are used as sole carbon source; weak growth occurs with glucose, maltose, and glycerol. Fructose, sucrose, ribose, arabinose, butyrate, formate, fumarate, propionate, benzoate, tartrate, methanol, ethanol, mannitol, and glycolate are not used. Gelatin, starch, and Tween-80 are not hydrolyzed.

The cells can oxidize thiosulfate to sulfate in the presence of organic compounds. Ribulose diphosphate carboxylase activity is not detected. The cells are susceptible to tetracycline, streptomycin, polymyxin-B, erythromycin, amikacin, kanamycin, neomycin, aureomycin, vancomycin, chloramphenicol, and fusidic acid, but resistant to gentamicin, lincomycin, nystatin, bacitracin, and penicillin. Oxidase and catalase positive.

Resistant to the heavy metal oxide tellurite. Reduction of tellurite results in the accumulation of elemental tellurium inside the cells. Tellurite resistance and reduction depend on supplemented organic carbon source. The storage material consists of polysaccharides, poly- β -hydroxybutyrate, and polyphosphate.

Habitat: freshwater cyanobacterial mat of a thermal alkaline sulfide spring (54°C; pH 9.3; 7.4 mg/l sulfide; 1.6 mg/l oxygen).

The mol% G + C of the DNA is: 70.4 (*T_m*).

Type strain: Strain RB3, ATCC 700004, DSM 8511.

GenBank accession number (16S rRNA): X72908.

Genus XIII. *Roseomonas* Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1998, 627^{VP}
(Effective publication: Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282)

ROBBIN S. WEYANT AND ANNE M. WHITNEY

Ro.se.o.mo' nas. M.L. adj. *roseus* rosy, rose-colored, or pink; Gr. n. *monas* a unit; M.L. n. *Roseomonas* a pink-pigmented bacterium.

Plump cocci, coccobacilli, or short rods. Arranged singly and less frequently in pairs or short chains. Gram negative. **Aerobic**. Growth occurs on trypticase soy agar with and without 5% sheep blood, heart infusion agar with and without 5% rabbit blood, chocolate agar, and buffered charcoal yeast extract (BCYE) agar. Most strains grow on MacConkey agar. Growth occurs at 25°C, 30°C, 35°C, and for most strains, 42°C. Optimal growth temperature is 35°C. No growth in media containing greater than 6% NaCl. Pinpoint colonies appear after 48–72 h incubation on BCYE agar. Colonies are raised, entire, glistening, and often mucoid. **Pale pink growth pigment is produced. Catalase and urease positive.** Oxidase variable. Motility variable. Indole is not produced. Associated with human infections, usually as a secondary or opportunistic pathogen. Interrelatedness of species by DNA–DNA hybridization is 7–53%.

The mol% G + C of the DNA is: 65–71.

Type species: *Roseomonas gilardii* Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1998, 627 (Effective publication: Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.)

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic treatment The genus *Roseomonas* contains three named species and three currently unnamed genomospecies: *R. gilardii*, *R. cervicalis*, *R. fauriae*, and *Roseomonas* genomospecies 4, 5, and 6. Fig. BXII.α.32 illustrates the phylogenetic relatedness among the species of *Roseomonas* based on 16S rDNA sequence similarity. As currently defined, *Roseomonas* is a dichotomous genus with one cluster of species, including *R. gilardii*, *R. cervicalis*, and *Roseomonas* genomospecies 4 and 5 in the family *Acetobacteraceae* and another cluster, including *R. fauriae* and *Roseomonas* genomospecies 6 in the family *Rhodospirillaceae*. Both *Roseomonas* clusters are in the *Alphaproteobacteria*.

Cell and flagellum morphology *Roseomonas* cells can be visualized using the Gram stain and other common staining techniques. Gram-stained preparations of 48-hour cultures show weakly staining Gram-negative plump coccoid rods, occasionally appearing in pairs or short chains. Cells of *R. fauriae* and *Roseomonas* genomospecies 6 have a more rod-like appearance than cells of the other species. Motility varies between species and between strains within species. Motile strains are found in all species except *Roseomonas* genomospecies 5. When present, motility is provided by a single polar flagellum per cell (Weyant et al., 1996).

Cellular fatty acid (CFA) composition *Roseomonas* species produce two characteristic CFA profiles that correlate with the dichotomy of the genus. The profile produced by *R. gilardii*, *R. cervicalis*, and *Roseomonas* genomospecies 4 and 5 is characterized by large amounts (43–53%) of *cis*-11-octadecenoic, δ -*cis*-11,12-methyleneoctadecanoic (10–25%), 2-hydroxyoctadecenoic (2–5%), and 2-hydroxy- δ -*cis*-11,12-methyleneoctadecanoic (3–11%) acids. The profile produced by *R. fauriae* and *Roseomonas* genomospecies 6 is characterized by very large amounts (63–90%) of *cis*-11-octadecenoic and small amounts of 3-hydroxytetradecanoic (2–6%), *cis*-9-hexadecenoic (3–12%), *cis*-9,12-octadeca-

dienoic, *cis*-9-octadecenoic, and octadecanoic acids (Wallace et al., 1990; Weyant et al., 1996).

Colonial and cultural characteristics Growth is achieved in aerobic conditions on most nonselective media, including trypticase soy agar with and without 5% sheep blood, heart infusion agar with and without 5% rabbit blood, chocolate agar, nutrient agar, Sabouraud dextrose agar, and buffered charcoal yeast extract (BCYE) agar. Most strains also grow on MacConkey and Thayer–Martin media. When cultured under optimal conditions, pinpoint colonies are observed after 48 hours of incubation at 35°C. Colonies are typically shiny, raised, entire, and often mucoid. Young colonies have a pale pink color that darkens with age.

Genetics The organization of the genus *Roseomonas* was determined in a DNA–DNA hybridization-based study of 42 strains by Rihs et al. (1993). Using the guidelines of the *ad hoc* committee on reconciliation of approaches to bacterial systematics (Wayne et al., 1987), six genomospecies, three named and three currently unnamed, were identified. The DNA mol% G + C contents of the six genomospecies range from 65.0 for *Roseomonas* genomospecies 6 to 70.4 for *R. cervicalis*. Lewis et al. (1997) have applied molecular subtyping approaches, including pulsed-field gel electrophoresis of chromosomal digests (PFGE) and random amplified polymorphic DNA (RAPD) analysis, to *R. gilardii* clinical isolates. RAPD analysis was reported to be more discriminatory than PFGE and less susceptible to interference by mucoid extracellular material produced by the isolates (Lewis et al., 1997).

Antibiotic sensitivity A significant proportion of the current *Roseomonas* literature contains information related to antibiotic susceptibility of *R. gilardii* strains (Gilardi and Faur, 1984; Korvick et al., 1989; Rihs et al., 1993; Lewis et al., 1997; Sandoe et al., 1997). Antibiotics to which all *R. gilardii* strains are susceptible include the aminoglycosides (gentamicin, amikacin, and tobramycin), tetracycline, and imipenem. One strain tested by Sandoe et al. (1997) was sensitive to netilmicin. All *R. gilardii* strains are resistant to piperacillin and erythromycin. The majority of strains tested were resistant to first- and second-generation cephalosporins. Susceptibility to third-generation cephalosporins varied within and between studies. The eight strains studied by Lewis et al. (1997) and two strains studied by Korvick et al. (1989) were susceptible to ceftriaxone, but only 1 of 23 strains studied by Rihs et al. (1993) was susceptible to this antibiotic. Likewise, the two strains studied by Korvick et al. (1989) were susceptible to cefotaxime but only 1 of 23 strains studied by Rihs et al. (1993) was susceptible. All strains studied were resistant to ceftazidime. Quinolone susceptibility also varied between studies. All eight strains studied by Lewis et al. (1997) and the strain studied by Sandoe et al. (1997) were susceptible to ciprofloxacin, but only 15 of 23 strains studied by Rihs et al. (1993) were susceptible. The study by Rihs et al. (1993) also included norfloxacin, to which 19 of 23 strains tested were susceptible. Susceptibility to sulfamethoxazole/trimethoprim and to penicillins (including natural, extended spectrum, and combinations with β -lactamase inhibitors) was variable in all studies that included more than

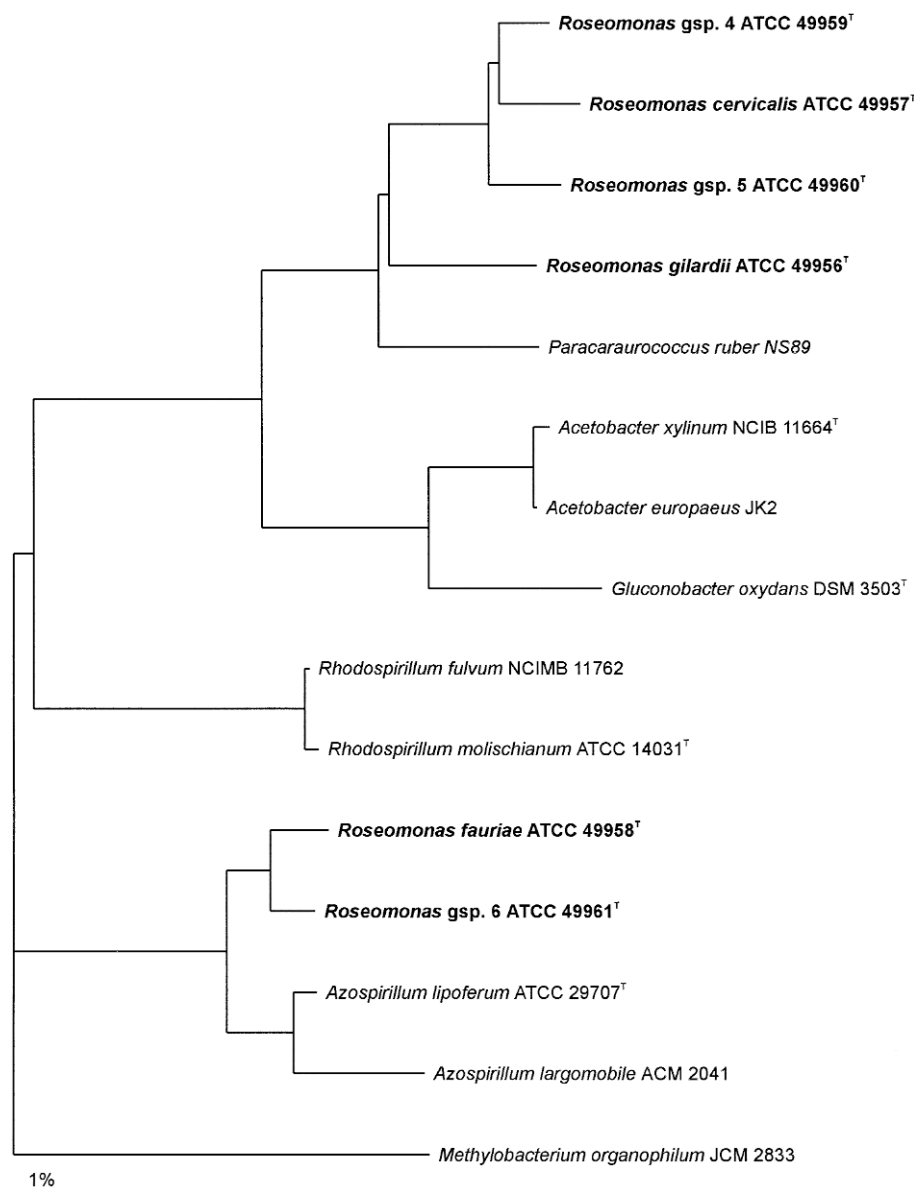


FIGURE BXII.α.32. Phylogenetic tree based on 16S rDNA sequence homologies between the type strains of *Roseomonas* species and type or reference strains of closely related taxa. *Acetobacter xylinum* and *Acetobacter europaeus* have been proposed as *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus* (Yamada et al., 1997, 1998b); *Rhodospirillum fulvum* and *Rhodospirillum molischianum* have been proposed as *Phaeospirillum fulvum* and *Phaeospirillum molischianum* (Imhoff et al., 1998).

one strain. Two strains tested by Korvick et al. (1989) were sensitive to chloramphenicol but resistant to clindamicin, vancomycin, and rifampin.

Rihs et al. (1993) also provided *in vitro* antibiotic susceptibility profiles for strains of the other *Roseomonas* species. All strains tested were sensitive to aminoglycosides, quinolones, tetracycline, imipenem, and penicillins in combination with β -lactamase inhibitors. Most strains were resistant to natural and extended spectrum penicillins, aztreonam, nitrofurantoin, and cephalosporins. Variable results were obtained for the sulfonamides.

Pathogenicity *Roseomonas* species have been associated with human infections, usually as secondary or opportunistic patho-

gens. There is currently no evidence that indicates *Roseomonas* species cause disease in plants or animals other than humans. Most *Roseomonas* infections have been reported in patients with significant underlying disease and, in many cases, *Roseomonas* strains were isolated in mixed culture. In a series of 35 cases reviewed by Struthers et al. (1996), *Roseomonas* strains were most commonly isolated from middle-aged women with one of several underlying conditions, including cancer and diabetes; blood was the most common source of isolation; and only 75% of strains were isolated in pure culture. Similar findings were reported by Lewis et al. (1997), who reviewed a series of eight cases of *R. gilardii* infection. All eight patients had underlying conditions, including ovarian cancer, AIDS, multiple myeloma, non-Hodg-

kin's lymphoma, and breast cancer. *R. gilardii* was isolated from blood cultures of seven patients; all of whom had central venous catheters and four of whom had polymicrobial infections. Based on the cases reported in the literature, *R. gilardii* is more likely to be associated with human infections than are the other *Roseomonas* species.

Ecology The ecology of *Roseomonas* has not been studied directly, with the great majority of known strains having been isolated from human clinical specimens. There are some suggestions in the literature that these organisms may reside in potable water. Of 42 *Roseomonas* strains studied by Rihs et al. (1993), three *R. gilardii* strains were isolated from potable water. Wallace et al. (1990) reported the source of 2 of the 156 strains that they studied to be a saline contaminant and ice. Another possible ecological niche for these organisms is human skin or mucosal surfaces. The type strain of *R. cervicalis* was isolated from a human cervix culture and *Roseomonas* genomospecies 5 has been isolated as a commensal from young adults attending a sexually transmitted diseases clinic (Rihs et al., 1993; Struthers et al., 1996).

ENRICHMENT AND ISOLATION PROCEDURES

Roseomonas species grow on most commonly used bacteriological media, including trypticase soy agar with and without 5% sheep blood, heart infusion agar with and without 5% rabbit blood, Sabouraud dextrose agar, nutrient agar, buffered charcoal yeast extract agar, and chocolate agar. Selective media that may be used to enrich for these organisms include Thayer–Martin agar and MacConkey agar, although a few MacConkey-negative strains have been described (Odugbemi et al., 1988; Weyant et al., 1996). Growth is inhibited in media containing more than 6% NaCl. Optimal growth temperature is 35°C and aerobic atmospheric conditions are satisfactory.

MAINTENANCE PROCEDURES

Strains can be maintained for short periods (up to 3 months) as cultures in motility deeps. Motility medium deeps in screw cap tubes are inoculated with a sterile inoculation needle by stabbing fresh growth approximately 5 cm into the agar with a single stroke. The tubes are incubated for 1–2 d at 35°C with the screw cap opened slightly to allow for air exchange. After growth is observed in the tube, the cap is tightened to prevent desiccation and the tube is stored at room temperature. Successful long-term storage can be achieved by suspending fresh growth in defibrinated rabbit blood and freezing in liquid nitrogen (Weyant et al., 1996).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

The oxidation–fermentation (OF) medium of King (Weyant et al., 1996) is more sensitive than the Hugh–Leifson formulation (Hugh and Leifson, 1953) in detecting acid production from carbohydrates with *Roseomonas* strains (Rihs et al., 1993). Long-wave absorbance of UV light is a relatively simple procedure for differentiating *Roseomonas* from *Methylobacterium* strains. Colonies grown on BCYE agar are exposed to a long wave UV lamp (365 nm) at a distance of approximately 5 inches (15 cm) in a dark room. *Methylobacterium* colonies will appear dark due to the absorbance of the light, whereas *Roseomonas* species will be easily visible due to the reflection of the light (Rihs et al., 1993). Interlab variability for the oxidase test with *R. gilardii* strains has been reported (Rihs et al., 1993). When tested by the Kovacs

method (Kovács, 1956), some strains may test negative at 10 seconds but will produce a weak reaction after 30 seconds.

DIFFERENTIATION OF THE GENUS *ROSEOMONAS* FROM OTHER GENERA

Roseomonas shares many phenotypic characteristics with *Methylobacterium*, a genus of aerobic, pink-pigmented, oxidative, Gram-negative rods. *Methylobacterium* strains have also been isolated from human clinical specimens (Weyant et al., 1996). Characteristics useful in differentiating these two genera are given in Table BXII.α.28. *Roseomonas* strains fail to produce acid from methanol, assimilate acetamide, and absorb long-wave (365 nm) UV light (Rihs et al., 1993). Most *Roseomonas* strains grow on MacConkey agar, although detectable growth may require 3–7 d of incubation. Esculin hydrolysis and motility are useful in differentiating strains of some *Roseomonas* species from *Methylobacterium*. Strains of *R. fauriae* and *Roseomonas* genomospecies 6 are esculin-positive, and strains of *Roseomonas* genomospecies 5 are nonmotile, which differentiate them from *Methylobacterium* strains.

TAXONOMIC COMMENTS

Strains subsequently shown to be *Roseomonas* were first described in a study of pink-pigmented clinical isolates by Gilardi and Faur. They designated their strains as an “unnamed pink-pigmented taxon” that could be differentiated phenotypically from *Methylobacterium* (Gilardi and Faur, 1984). This term was used by Odugbemi et al. and Korvick, et al. to describe additional isolates from human blood cultures (Odugbemi et al., 1988; Korvick et al., 1989). In 1990 Wallace, et al. published an extensive phenotypic and cellular fatty acid analysis of 156 pink-pigmented strains submitted to the U.S. Centers for Disease Control and Prevention for identification. Included within the study group were the strains described by Odugbemi et al. and by Gilardi and Faur. Four phenotypic groups (Pink Coccoid Groups I, II, III, and IV) that were differentiated by esculin hydrolysis, oxidation of D-xylose and D-mannitol, and cellular fatty acid profiles were described (Wallace et al., 1990). In 1993, Rihs et al., published a polyphasic classification study of 42 strains, including the strains from Gilardi and Faur, Odugbemi et al., Korvick et al., and representatives of all four Pink Coccoid Groups from Wallace et al. Using DNA–DNA hybridization, in conjunction with morphological, biochemical, and antibiotic susceptibility profile analysis, the genus containing six new species (*Roseomonas gilardii*, *R. cervicalis*, *R. fauriae*, and *Roseomonas* genomospecies 4, 5, and 6) was described (Rihs et al., 1993). Recently obtained 16S rDNA sequence information now suggests phylogenetic dichotomy within the genus *Roseomonas* (Fig. BXII.α.32). The type species, *R. gi-*

TABLE BXII.α.28. Differentiation of *Roseomonas* species from *Methylobacterium* species^{a,b}

Characteristic	<i>Roseomonas</i> species	<i>Methylobacterium</i> species
Acid from methanol	—	+
Assimilation of acetamide	—	+
Absorption of long-range UV light	—	+
Growth on MacConkey agar	+ ^c	—
Esculin hydrolysis	d	—
Motility	d	+

^aFor symbols see standard definitions.

^bData from Rihs et al. (1993) and Weyant et al. (1996).

^cSome strains require 3–7 days for detection of growth.

lardii, along with *R. cervicalis* and *Roseomonas* genomospecies 4 and 5 are mostly closely related to taxa in the family *Acetobacteraceae*. *R. fauriae* and *Roseomonas* genomospecies 6, however, are more closely related to taxa in the family *Rhodospirillaceae*. Cellular morphology (more rod-like for *R. fauriae* and *Roseomonas* genomospecies 6), CFA profile analysis, and intraspecies DNA-DNA hybridization findings also suggest a dichotomy between the species clustered around *R. gilardii* and those clustered around *R. fauriae* (Rihs et al., 1993; Weyant et al., 1996).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ROSEOMONAS*

Biochemical tests useful in the differentiation of *Roseomonas* species are given in Table BXII.α.29. *R. fauriae* and *Roseomonas* genomospecies 6 are the only *Roseomonas* species that contain strains which test positive for esculin hydrolysis. The ability to produce acid from L-arabinose, D-galactose, and D-xylose differentiates *R. fauriae* from *Roseomonas* genomospecies 6. Acidification of D-mannose differentiates *Roseomonas* genomospecies 4

FURTHER READING

- Gilardi, G.L. and Y.C. Faur. 1984. *Pseudomonas mesophilica* and an unnamed taxon, clinical isolates of pink-pigmented oxidative bacteria. *J. Clin. Microbiol.* 20: 626-629.
- Rihs, J.D., D.J. Brenner, R.E. Weaver, A.G. Steigerwalt, D.G. Hollis and V.L. Yu. 1993. *Roseomonas*, a new genus associated with bacteremia and other human infections. *J. Clin. Microbiol.* 31: 3275-3283.
- Wallace, P.L., D.G. Hollis, R.E. Weaver and C.W. Moss. 1990. Biochemical and chemical characterization of pink-pigmented oxidative bacteria. *J. Clin. Microbiol.* 28: 689-693.

from the other esculin-negative species. Motility and glycerol oxidation are useful in differentiating between *R. gilardii*, *R. cervicalis*, and *Roseomonas* genomospecies 5. All *Roseomonas* genomospecies 5 strains studied thus far have been negative for either citrate alkalization or acid production from glycerol, which differentiates this species from *R. gilardii* (Rihs et al., 1993).

List of species of the genus *Roseomonas*

1. ***Roseomonas gilardii*** Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1998, 627^{VP} (Effective publication: Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.) *gi.lar' di.i*. M.L. gen. n. *gilardii* named after Gerald L. Gilardi, who first described these organisms in 1978.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This species has been associated with opportunistic infections of humans, including bacteremia. Although a few strains have been isolated from potable water, the natural reservoir of this species is unknown.

The mol% G + C of the DNA is: 67.6-71.2 (T_m).

Type strain: 5424, ATCC 49956, CIP 104026.

2. ***Roseomonas cervicalis*** Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1998, 627^{VP} (Effective publication: Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.) *cervi.ca' lis*. M.L. adj. *cervicalis* from the cervix.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This species has been associated with urogenital and eye infections of humans.

The mol% G + C of the DNA is: 70.4 (T_m).

Type strain: E7107, ATCC 49957, CIP 104027.

3. ***Roseomonas fauriae*** Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1998, 627^{VP} (Effective publication: Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.) *faur' i.a.e*. M.L. n. *fauriae* named after Yvonne Faur, who first described *Roseomonas* isolates in 1978.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This species has been associated with bacteremia and wound infections of humans.

The mol% G + C of the DNA is: 68 (T_m).

Type strain: C610, ATCC 49958, CIP 104028.

TABLE BXII.α.29. Characteristics useful in differentiating *Roseomonas* species^{a,b}

Characteristic	<i>R. gilardii</i>	<i>R. cervicalis</i>	<i>R. fauriae</i>	<i>Roseomonas</i> genomospecies 4	<i>Roseomonas</i> genomospecies 5	<i>Roseomonas</i> genomospecies 6
Esculin hydrolysis	—	—	+	—	—	+
Citrate alkalization	+	d	d	—	d ^c	+
Motility	d	+	+	d	—	+
Acid production from:						
L-Arabinose	d	d	+	d	d	—
D-Galactose	d	—	+	d	d	—
Glycerol	+	—	+	—	d ^b	+
D-Mannose	—	—	—	+	—	—
D-Xylose	d	d	+	+	d	—

^aFor symbols see standard definitions.

^bData from Rihs et al. (1993) and Weyant et al. (1996).

^cAll *Roseomonas* genomospecies 5 strains studied thus far have been negative for either citrate alkalization or acid production from glycerol (Rihs et al., 1993).

TABLE BXII.α.30. Biochemical characteristics of *Roseomonas* species^{a,b}

Characteristic	<i>R. gilardii</i>	<i>R. cervicalis</i>	<i>R. fauriae</i>	<i>Roseomonas</i> genomospecies 4	<i>Roseomonas</i> genomospecies 5	<i>Roseomonas</i> genomospecies 6
Pink growth pigment	+	+	+	+	+	+
Absorption of UV light (365 nm)	—	—	—	—	—	—
Motility	d	+	+	d	—	+
Growth at:						
25°C	+	+	+	+	+	+
35°C	+	+	+	+	+	+
42°C	d	+	+	+	d	+
Growth on:						
MacConkey agar ^c	+	+	+	+	+	+
<i>Salmonella</i> – <i>Shigella</i> agar	—	—	d	—	—	—
Cetrimide agar	—	—	—	—	—	—
Growth in nutrient broth	+	+	+	+	+	+
Growth in nutrient broth with 6% NaCl	d	—	d	d	—	—
Catalase	+	+	+	+	+	+
Oxidase	v ^d	+	+	+	+	+
Simmons citrate	+	+	d	—	d	+
Christensen's urea	+	+	+	+	+	+
Nitrate reduction	—	—	+	+	—	+
Gas from nitrate	—	—	d	—	—	—
Indole	—	—	—	—	—	—
Esculin hydrolysis	—	—	+	—	—	+
H ₂ S production (TSI butt) ^e	—	—	—	—	—	—
<i>o</i> -Nitrophenyl-β-D-galactopyranoside	—	—	—	—	—	—
Phenylalanine deaminase	—	—	—	—	—	—
L-Lysine decarboxylase	—	—	—	—	—	—
L-Arginine dihydrolase	—	—	—	—	—	—
L-Ornithine decarboxylase	—	—	—	—	—	—
Acid production from:						
D-Glucose	d	—	d	—	—	—
D-Xylose	d	d	+	+	d	—
D-Mannitol	d	—	—	—	—	—
Lactose	—	—	—	—	—	—
Sucrose	—	—	—	—	—	—
Maltose	—	—	—	—	—	—
D-Mannose	d	—	—	+	—	—
L-Arabinose	d	d	+	d	d	—
Fructose	+	+	+	d	+	+
D-Galactose	d	—	+	d	d	—
Salicin	—	d	d	—	—	+
Dulcitol	—	—	—	—	—	—
Methanol	—	—	—	—	—	—
Raffinose	—	—	—	—	—	—
L-Rhamnose	—	—	—	—	—	—

^aFor symbols see standard definitions.^bData from Rihs et al. (1993) and Weyant et al. (1996).^cSome strains require 3–7 days for detection of growth.^dInterlab variability has been reported for testing *R. gilardii* strains by the method of Kovacs (Kovács, 1956); some strains may produce a weak and extremely delayed reaction (Rihs et al., 1993).^eTSI, triple sugar iron agar.

Other Organisms

1. *Roseomonas* genomospecies 4 Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This genomospecies has been isolated from human wound, ear, and cervix specimens.

The mol% G + C of the DNA is: 67.8 (*T_m*).

Deposited strain: E7832, ATCC 49959.

2. *Roseomonas* genomospecies 5 Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This genomospecies has been isolated from human blood, bone tissue, and breast tissue.

The mol% G + C of the DNA is: 65 (*T_m*).

Deposited strain: F4700, ATCC 49960.

3. *Roseomonas* genomospecies 6 Rihs, Brenner, Weaver, Steigerwalt, Hollis, and Yu 1993, 3282.

The morphological and cultural characteristics are as described for the genus. Other characteristics of this species are given in Table BXII.α.30.

This genomospecies has been isolated from human wound material.

The mol% G + C of the DNA is: 65.4 (*T_m*).

Deposited strain: F4626, ATCC 49961.

Genus XIV. *Stella* Vasilyeva 1985, 520^{VP}

LEANA V. VASILYEVA

Stella M.L. fem. n. *Stella* star, to denote star-shaped morphology of cells.

Cells are flat, six-pronged stars, radially symmetrical, and 0.7–3.0 μm . Occur singly or in pairs. Some strains possess **gas vesicles**. Spores are not formed. **Gram negative**. Aerobic. Reproduction is by **symmetrical cell division**.

Chemoorganotrophic, using a variety of amino acids or organic acids. Aerobic and oxidative. **Oligocarboxiphilic**.

Occur in soil, freshwaters, and artificial ecosystems where complete decomposition of organic matter is underway; typical representative of the microflora of dispersal systems.

The mol% G + C of the DNA is: 69.3–73.5 (T_m).

Type species: ***Stella humosa*** Vasilyeva 1985, 520.

FURTHER DESCRIPTIVE INFORMATION

Flat, star-shaped bacteria were first observed by Nikitin and colleagues (Nikitin et al., 1966) and shortly thereafter by Staley (Staley, 1968). Since that time, these prosthecobacteria have been discussed in a number of publications (Vasilyeva, 1970, 1972b, 1980, 1984, 1986; Vasilyeva et al., 1974; Hirsch et al., 1977; Stanley et al., 1979; Hirsch and Schlesner, 1981; Staley et al., 1981; Terkhova et al., 1981; Schlesner, 1983; Reimer and Schlesner, 1989).

The characteristic morphology of *Stella* species is that of a flat, six-pointed star ("Star of David"). There are also intermediate forms among the flat prosthecobacteria, such as the flat triangles observed in organisms assigned to the genus *Labrys* (Vasilyeva and Semenov, 1984), and the genus *Angulomicrobium* (Vasilyeva et al., 1979).

The reproduction of star-shaped bacteria is quite similar in all strains studied. Cell division proceeds by cross-wall formation along a line where the cell has the smallest diameter and between opposite pairs of prongs. Both daughter cells retain three prongs each from the mother cell and then form three new additional prongs (Vasilyeva, 1972b). The cell cytoplasm appears to be similar to that of other Gram-negative bacteria, and so far, no obvious intracellular membrane component that might account for the prong structures that occur on the flat cells of the organism has been observed. Poly- β -hydroxybutyrate storage granules are apparent within some cells. Morphology of the star-shaped forms can vary with changes in concentration of nutrient in the broth, as well as within different strains.

The phylogenetic position of the genus *Stella* indicates that the star-shaped organism belongs to the *Alphaproteobacteria* (Fischer et al., 1985; Stackebrandt et al., 1988a, b; Stackebrandt, et al., 1988a).

Strains of the genus *Stella* are able to utilize a variety of organic acids or amino acids as substrates. The majority of strains utilize pyruvate, citrate, α -ketoglutarate, succinate, malate, acetate, and glutamate. Some strains additionally utilize lysine, glutamine, cysteine, cystine, L-alanine, asparagine, aspartate, and gluconate, and only one or a few strains utilize fumarate, butyrate, valerate, D-alanine, arginine, proline, threonine, or histidine. None of the strains utilizes propionate, benzoate, urea, ethanol, methanol, monomethylamine, leucine, isoleucine, methionine, valine, tryptophan, phenylalanine, glycine, or oxyproline. Complex carbohydrate or protein polymers, cellulose, starch, and gelatin are

not catabolized. In addition to the compounds listed, star-shaped bacteria require 0.01% yeast extract or Casamino acids in the growth medium. A mixture containing 0.1% L-glutamic acid and various B vitamins can be substituted for the yeast extract. Star-shaped bacteria are obligately aerobic, and their metabolism is oxidative (Vasilyeva et al., 1974).

The strains of *Stella* were obtained from a variety of soil, aquatic, and animal fecal materials (Hirsch and Schlesner, 1981; Vasilyeva, 1985; Schlesner, 1992).

From ecological observations performed with direct microscopic techniques and enrichment broth cultures, it is apparent that these organisms are found in habitats where active degradation of organic substances is occurring. Thus, these prosthecobacteria are typical representatives of the aerobic oligotrophic microflora of dispersal (Zavarzin, 1970), now designated as dissipotrophs (Vasilyeva and Zavarzin, 1995), which are defined as an ecological group of bacteria that utilize low molecular weight compounds formed by other microorganisms during hydrolysis of particulate organic matter.

ENRICHMENT AND ISOLATION PROCEDURES

The most successful method for recovery of *Stella* species involves the use of enrichment cultures containing prosthecobacteria growing in association with aerobic cellulose-decomposing bacteria on Hutchinson medium. The unique morphology of these star-shaped bacteria is usually obvious under phase-contrast microscopy. Colonies on agar plates are usually mixed, and a long series of subculturing and reisolation of single colonies is required for selection of pure cultures.

Pure cultures of organisms can be maintained either in dilute meat-peptone media (1:5), such as nutrient broth with beef extract and peptone added and solidified with 2% (w/v) agar, or in media described by Staley (1989). This defined medium supplemented with 0.01% yeast extract can be used for determination of carbon source utilization.

MAINTENANCE PROCEDURES

Strains can be maintained on slants in the refrigerator for at least 6 months. Lyophilization can be used for long-term preservation.

DIFFERENTIATION OF THE GENUS *STELLA* FROM OTHER GENERA

The cluster of star-shaped bacteria appears to represent a clear and distinct group within the collection of budding and/or appendaged bacteria. The mode of cell division, occurring as a cross-wall separating two equal daughter cells with a profile of two three-pronged flat crowns, provides a major means to separate organisms in the genus *Stella* from other prosthecobacteria, such as *Hyphomicrobium*, *Hyphomonas*, *Pedomicrobium*, *Rhodomicrobium*, *Ancalomicrobium*, *Ancalochloris*, *Prosthecomicrobium*, and *Prosthecochloris*. The flat cellular morphology of *Stella* is shared with the genus *Labrys*, but the latter multiplies by budding. DNA–DNA homology between *Stella* species and *Labrys* is quite low.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *STELLA*

The two species, *S. humosa* and *S. vacuolata*, of the genus *Stella* are differentiated by the presence or absence of gas vesicles,

which correlates with a number of other characters, such as substrate utilization and a slightly different mol% G + C content

of their DNA. All vacuolate strains had DNA–DNA homologies in the range of 74–100%; however, some nonvacuolated strains had DNA–DNA homologies in the range of 60–72% while others had homologies as low as 3%, indicating that stellas are hetero-

ogeneous. Two additional species, “*S. aquatica*” and “*S. pusilla*”, were described by Schlesner (Schlesner, 1983); differentiating characters, however, remain unclear.

List of species of the genus *Stella*

1. *Stella humosa* Vasilyeva 1985, 520^{VP}

hu.mo'sa. M.L. fem. adj. *humosa* soil, earth.

Cells are flat, six-pronged stars 0.7–3.0 µm in diameter and occur singly or in pairs (Fig. BXII.α.33). No clusters or other aggregates are formed. Gram negative with typical three-layered cell envelope. Nonmotile. Multiplication by symmetrical cell division. Aerobic. Poly-β-hydroxybutyrate granules are formed as the reserve substance.

Colonies are grayish white, circular, compact, and up to 2.5 mm in diameter after incubation for 14 d at 28°C.

Chemoorganotrophic, utilizing as sole energy source a limited number of organic acids of the tricarboxylic acid cycle and amino acids for respiration. Carbohydrates are not fermented or utilized. Yeast extract is required by all strains but may be replaced by glutamic acid and B vitamins in some strains. No hydrolytic activity. Polysaccharides and proteins not degraded. Oligocarbophilic.

Optimal temperature for growth is 28–30°C. Optimal pH is neutral for the type strain and is slightly alkaline for others. Growth of some strains is stimulated by the addition of up to 1% NaCl. Catalase and oxidase positive.

Sensitive to neomycin (0.5–1.0 µg/ml); moderately sensitive to penicillin (8 µg/ml for type strain) and monomycin (2–10 µg/ml) as well as to most other antibacterial antibiotics (Terekhova et al., 1981).

Plasmids have so far not been detected.

The mol% G + C of the DNA is: 69.3–72.9 (T_m).

Type strain: AUCM B-1137, ATCC 43930, DSM 5900.

2. *Stella vacuolata* Vasilyeva 1985, 521^{VP}

va'cu.o.la.ta. L. adj. *vacuus* empty, void; L. adj. *latus* broad, wide; M.L. adj. *vacuolata* large areas in cytoplasm that appear empty due to gas vesicle formation.

Cells are flat, six-pronged stars 1.9–2.5 µm in diameter. Occur singly or in pairs. Clusters or aggregates are not formed. Gram negative, nonmotile. Multiplication by symmetrical division into two initially three-pronged daughter cells. Distinctive morphological feature is the presence of gas vesicles throughout cell cytoplasm (Fig. BXII.α.34). Cells exhibit considerable buoyancy.

Colonies on agar prepared with dilute meat-peptone media (1:5) are milky white, circular and viscous and may reach 2.5 mm in diameter.

Growth in liquid broth: A pellicle is formed and sediment occurs on the bottom of tube. Growth is inhibited by shaking cultures and by 1% NaCl.

Chemoorganotrophic, aerobic. Selected amino acids and organic acids of the tricarboxylic acid cycle are utilized as energy sources. Yeast extract is required for growth, although Casamino acids or L-glutamic acid and a B vitamin mixture can substitute for yeast extract. Carbohydrates are

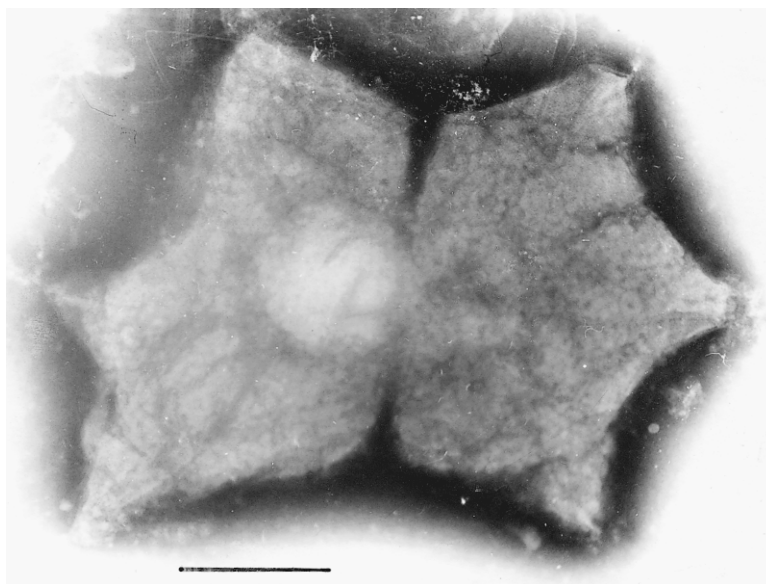


FIGURE BXII.α.33. *Stella humosa* type strain AUCMB-1137. Phosphotungstic acid-negative stain. Bar = 0.5 µm. (Reprinted with permission from E.N. Mischustin, Izvestiya Akademii Nauk SSSR. Seriya Biologicheskaya 5: 730, 1980, ©Izdatel'stvo Nauka, Moscow, Russia.)

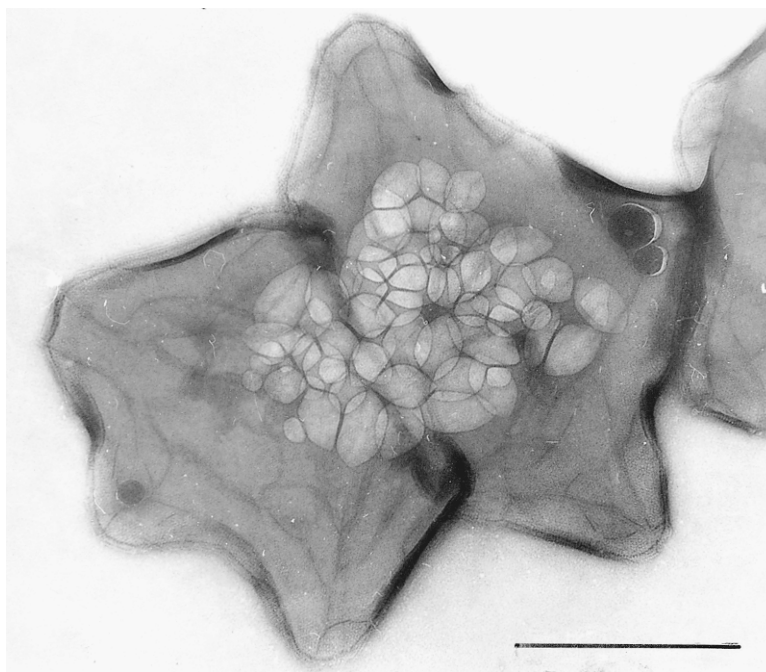


FIGURE BXII.α.34. *Stella vacuolata* Bright areas in cytoplasm are gas vesicles. Uranylacetate-negative stain. Bar = 1.0 μm.

not utilized but do not inhibit growth. Do not possess the ability to utilize polymers.

Optimal temperature for growth: 28°C. Optimal pH for growth: neutral or slightly alkaline. Plasmids were not detected.

Habitat: isolated from horse manure and sewage sludge from a piggery.

The mol% G + C of the DNA is: 70–73.5 (T_m).

Type strain: AUCM B-1552, ATCC 43931, DSM 5901.

Genus XV. Zavarzinia Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 393)

THE EDITORIAL BOARD

Za.var.zi'ni.a. M.L. n. *Zavarzinia* named for Georgi Alexandrovich Zavarzin, the Russian microbiologist who with his co-workers made the first thorough investigation of mesophilic, Gram-negative carboxidotrophic strains.

The genus description is the same as the description for *Zavarzinia compransoris*.

The mol% G + C of the DNA is: 66.1.

Type species: ***Zavarzinia compransoris*** (ex Nozhevnikova and Zavarzin 1974) Meyer, Stackebrandt and Auling 1994, 182 (Effective publication: Meyer, Stackebrandt and Auling 1993, 393.)

List of species of the genus *Zavarzinia*

1. ***Zavarzinia compransoris*** (ex Nozhevnikova and Zavarzin 1974) Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 393.)
com.pran'so.ris. L. *compransoris* of a dinner companion.

Gram-negative, curved rod. Motile by means of a polar flagellum, 6–8.5 μm in length. Colonies are thin and translucent. Cells swarm on wet agar surfaces. Catalase positive. Thiamine required for growth. "*Pseudomonas gazotropha*" provides thiamine in mixed cultures. Utilizes organic acids

(except citrate) and amino acids as sole carbon and energy sources. No growth on sugars. Under aerobic conditions, utilizes ammonia, nitrate, nitrite, hydroxylamine, and urea as nitrogen sources. Heterotrophic denitrification occurs with pyruvate as the electron donor and forming N₂O. Chemolithoautotrophic growth with CO, with a doubling time of 18 h, and incorporation of 4% of the CO oxidized into cell carbon. Possesses cytochromes *a*, *b*, and *c*, but not *b*₅₆₃.

The mol% G + C of the DNA is: 66.1.

Type strain: ATCC 51430, DSM 1231, LMG 5821, LMG 8357, Z-1155.

Order II. **Rickettsiales** Gieszczykiewicz 1939, 25^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2156

J. STEPHEN DUMLER AND DAVID H. WALKER

Rick.ett.si.a'les. M.L. fem. n. *Rickettsia* type genus of the order; *-ales* ending to denote order; M.L. fem. pl. n. *Rickettsiales* the *Rickettsia* order

Rod-shaped, coccoid or irregularly shaped bacteria with typical Gram-negative cell walls and no flagella. Multiply only inside host cells. Can be cultivated in living tissues such as those of embryonated chicken eggs and metazoan cell cultures. All are regarded as parasitic or mutualistic. The bacteria are parasitic forms associated with host cells of the mononuclear phagocyte system, the hematopoietic system, or the vascular endothelium of vertebrates; with various organs and tissues of helminths; or with tissues of arthropods, which may act as vectors or primary hosts. May cause disease in man or in other vertebrate or invertebrate hosts. Mutualistic forms in insects and helminths may be required for development and reproduction of the host under some circumstances.

The mol% G + C of the DNA is: 28–56.

Type genus: **Rickettsia** da Rocha-Lima 1916, 567.

TAXONOMIC COMMENTS

In the ninth edition of *Bergey's Manual of Determinative Bacteriology*, Section 9, the order *Rickettsiales* was divided into three families, *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*. The recent removal of the family *Bartonellaceae* as well as some genera and species in the family *Anaplasmataceae* from the order also removes all species that could be propagated extracellularly—either on artificial media or on the surface of host cells. In addition, modern molecular classification methods and the recognition of shared biological and morphological features have allowed a reclassification of some *Rickettsiaceae* genera and species into the family *Anaplasmataceae*. These methods have also allowed the removal of some genera and species from *Rickettsiaceae* and *Anaplasmataceae* as well as the removal of the tribe structure of the family *Rickettsiaceae*. Currently, the order contains only the families *Rickettsiaceae* and *Anaplasmataceae*.

Family I. **Rickettsiaceae** Pinkerton 1936, 186^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2156

XUE-JIE YU AND DAVID H. WALKER

Rick.ett.si.a'ce.ae. M.L. fem. n. *Rickettsia* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Rickettsiaceae* the *Rickettsia* family.

Mainly **diplococcus-shaped, but can also be rod shaped or coccoid**. Gram negative. **Obligately intracellular. Intimately associated with arthropod hosts.** No flagella or endospores occur. No member of the family has yet been cultivated in cell-free media. Some species can be parasitic in man and other vertebrates, causing diseases (e.g., typhus and related illnesses in man) that are transmitted by arthropods (lice, fleas, ticks and mites). Some are confined to the invertebrate host as pathogens or symbionts.

The mol% G + C of the DNA is: 29–33.

Type genus: **Rickettsia** da Rocha-Lima 1916, 567.

TAXONOMIC COMMENTS

Historically, the designation “rickettsia” has been used indiscriminately for many small rods that could not be cultivated and were not otherwise identified. Most frequently, but not always, these organisms were seen in association with arthropods. The last edition of the *Manual of Systematic Bacteriology* (1st edition, 1984) reflects the considerable progress that has been made in establishing a more precise definition of the family *Rickettsiaceae* and

in eliminating species that do not fit the definition. The process continues with this edition. In the last edition, the family *Rickettsiaceae* included three tribes: *Rickettsieae*, *Ehrlichieae*, and *Wolbachieae*. In this edition, the tribes are eliminated. The tribe *Rickettsieae* contained three genera: *Rickettsia*, *Rochalimaea*, and *Coxiella*. The genus *Rickettsia* is retained in the family *Rickettsiaceae*. Neither *Rochalimaea* nor *Coxiella* are genotypically related to *Rickettsia* except for a superficial phenotypic similarity between *Rickettsia* and *Coxiella*. *Rochalimaea* has been combined with *Bartonella* and removed from the family *Rickettsiaceae* (Brenner et al., 1993). *Coxiella* has been removed from the family *Rickettsiaceae* and placed in the order “*Legionellales*”. Organisms in tribes *Ehrlichieae* and *Wolbachieae* have been removed from the family *Rickettsiaceae* and combined into the family *Anaplasmataceae*. *Rickettsia tsutsugamushi* was placed in a new genus *Orientia* as *Orientia tsutsugamushi* (Tamura et al., 1995). At present the family *Rickettsiaceae* consists of two genera: *Rickettsia* and *Orientia*. All organisms in the family are phylogenetically related (Fig. BXII.α.35).

Genus I. **Rickettsia** da Rocha-Lima 1916, 567^{AL}

XUE-JIE YU AND DAVID H. WALKER

Rick.ett'si.a. M.L. fem. n. *Rickettsia* named after Howard Taylor Ricketts, who first associated organisms of this description with spotted fever and typhus and who died of typhus contracted in the course of his studies.

Short, often paired rods, 0.3–0.5 × 0.8–2.0 μm. The rickettsial envelope has a typical Gram-negative structure with a bilayer inner membrane, a peptidoglycan layer, and a bilayer outer mem-

brane. The cells are often surrounded by a protein microcapsular layer and slime layer. Rickettsiae retain basic fuchsin when stained by the method of Giménez (1964). The organisms are

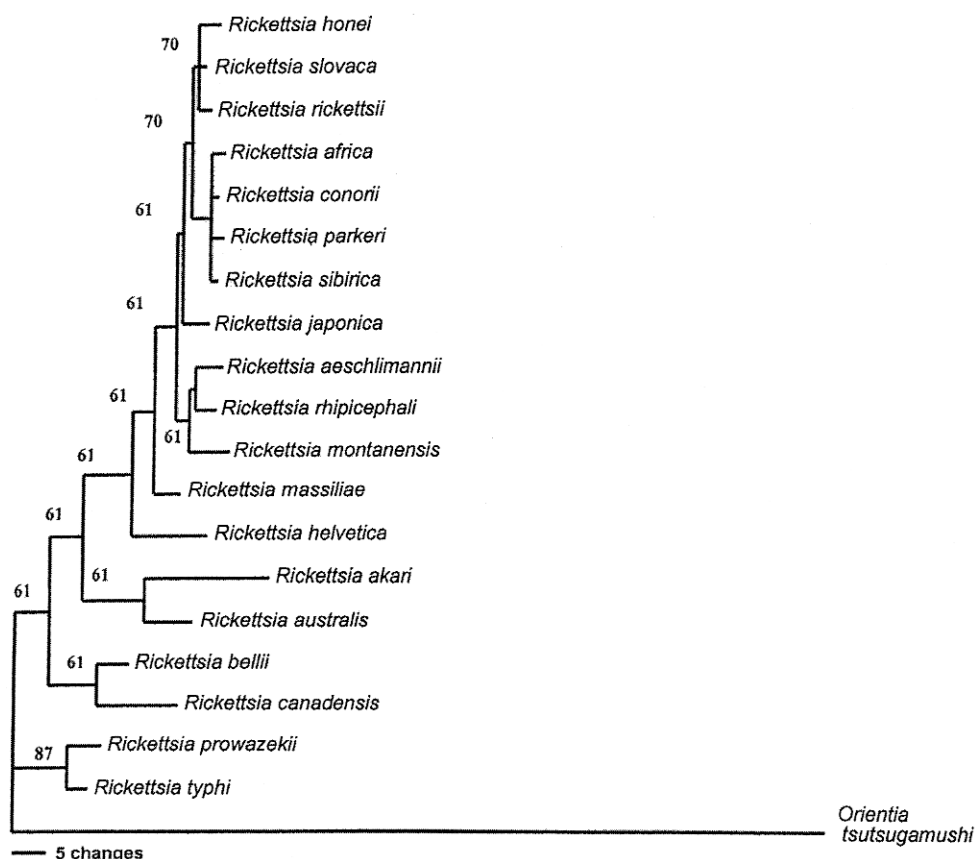


FIGURE BXII.α.35. Phylogenetic relationships of the organisms in the family *Rickettsiaceae* based on the DNA sequences of the 16S rRNA genes (GenBank accession numbers: *R. aeschlimannii*, RAU74757; *R. africae*, RIRRGDA; *R. akari*, RAU12458; *R. australis*, RAU17644; *R. bellii*, RBU11014; *R. canadensis*, RCU15162; *R. conorii*, RIRRGDH; *R. helvetica*, RIRRGDK; *R. honei*, AF060705; *R. japonica*, RIRRGDL; *R. massiliae*, RIRRGDI; *R. montanensis*, RIRRGDN; *R. parkeri*, RIRRRDA; *R. prowazekii*, RIRRGSA; *R. rhipicephali*, RIRRGDO; *R. rickettsii*, RIRRGDP; *R. sibirica*, RIRRS16SRG; *R. slovaca*, RIRRGDX; *R. typhi*, RIRRGDU; *O. tsutsugamushi*, RIRRTKP16B). The length of each pair of branches represents the distance between sequence pairs. The numbers on the branch indicate the bootstrap values.

obligately intracellular and reside free in the cytoplasm of the eucaryotic host cell, where they divide by **binary fission**. Rickettsiae of the spotted fever group (SFG) may also reside in the nucleus of the eucaryotic host cells. **Rickettsiae are closely associated with arthropods** (ticks, mites, fleas, lice, and other insects) **for their maintenance in nature**. Their natural cycle usually involves both a vertebrate and an invertebrate host. For some, the arthropod host is both a reservoir and a vector. **Transovarian transmission** of the agent from the infected female to the next generation is the essential mechanism for the maintenance of many species (Burgdorfer, 1988). Rickettsial cells are usually unstable when separated from host components, except for highly stable forms found in the feces of arthropod hosts; stability can be enhanced by certain proteins, sucrose, and reagents that tend to maintain the integrity of outer membranes, osmolarity, and ATP level. Rickettsiae are best preserved by rapid freezing and storage below -50°C . The cells are rapidly inactivated at 56°C . Rickettsiae derive energy from the metabolism of glutamate via the citric acid cycle, but do not utilize glucose. They transport and metabolize phosphorylated compounds but do not synthesize or degrade nucleoside monophosphates. **Rickettsiae are etiological agents of typhus and spotted fevers in humans**. There are 21 recognized species.

The mol% G + C of the DNA is: 29–33.

Type species: ***Rickettsia prowazekii*** da Rocha-Lima 1916, 567 (Nom. Cons. Opin. 19, Jud. Comm. 1958, 158.)

FURTHER DESCRIPTIVE INFORMATION

Phylogeny In the 1st edition of *Bergey's Manual of Systematic Bacteriology*, the organisms in the genus *Rickettsia* were divided into the typhus group (TG) and the spotted fever group (SFG). *R. prowazekii*, *R. typhi*, and *R. canadensis* were included in the TG; these species stimulate antibodies that react more strongly with *Proteus vulgaris* OX19 antigen than OX2 antigen and share LPS antigens. The other rickettsiae were included in the SFG; they stimulate antibodies that react more strongly with antibody specific for *Proteus vulgaris* OX2 antigen and share their own LPS antigens.

In the present edition, *Rickettsia* species are divided into three groups based on phylogeny (Fig. BXII.α.35), as follows. The TG includes *R. prowazekii* and *R. typhi*. The SFG includes *R. aeschlimannii*, *R. africae*, *R. akari*, *R. australis*, *R. conorii*, *R. felis*, *R. helvetica*, *R. honei*, *R. japonica*, *R. massiliae*, *R. montanensis*, *R. parkeri*, *R. peacockii*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, and *R. slovaca* (Bouyer et al., 2001). The third group, the ancestral group, includes *R. bellii*, *R. canadensis*, and the AB male-killing bacterium;

this group represents early divergent lineages within the genus (Stothard et al., 1994).

The numbers of named SFG species have increased markedly since the last edition owing to the improvement of isolation approaches and molecular methods for identification. However, there is no consensus concerning the criteria used to define a species of *Rickettsia*. The 16S rRNA gene sequences show 97.2–99.9% similarity among the SFG rickettsiae. The 16S rRNA gene sequences are $\geq 99.7\%$ similar among the following *Rickettsia* species: *R. africae*, *R. conorii*, *R. massiliae*, *R. parkeri*, *R. sibirica*, *R. slovaca*, and *R. rickettsii*. Thus, many designated species of SFG rickettsiae actually might be clones or strains of a single species according to the standards used for classifying other bacteria. In this chapter, we have compiled information about all *Rickettsia* species that were either included in the Approved Lists of Bacterial Names validly published after January 1980 regardless of where they stand in phylogeny. Rickettsiae need to be classified in the future by an authorized committee according to defined criteria.

Cell structure Although rickettsiae are relatively small bacteria, they closely resemble other Gram-negative bacteria. In smears from yolk sac, tissue, or cell culture, rickettsiae are best visualized by the Giménez (1964) stain. By this procedure, rickettsiae stain bright red with basic fuchsin, while the background is decolorized and stains a pale greenish blue with the malachite green counterstain (Fig. BXII.α.36A and B).

The rickettsial cell wall contains peptidoglycan, which consists of glutamic acid, alanine, and diaminopimelic acid in a molar ratio of 1.0:2.3:1.0. The small amount of lysine found in the peptidoglycan preparation suggests that a peptidoglycan-linked lipoprotein(s) might be present (Pang and Winkler, 1994).

In ultrathin sections viewed by electron microscopy, rickettsiae are surrounded by a typical Gram-negative envelope (Fig. BXII.α.36E–G). The rickettsial cytoplasm contains ribosomes and strands of DNA. Organisms of the genus *Rickettsia* are typically surrounded in the host cell by an electron-lucent zone that has been proposed to represent a slime layer, which is stabilized by the presence of antibodies (Fig. BXII.α.36E). During the course of infection, rickettsial morphology can change: older cells can become smaller and more electron dense. In *R. prowazekii*, cells may stop dividing after prolonged cultivation and become 3–4 μm long. They also may contain translucent vacuole-like structures, which in some cases appear to occupy as much as 25% of the cytoplasm (Wisseman and Waddell, 1975; Silverman et al., 1980). Intracytoplasmic crystalline structures may also be formed, presumably by a DNA-binding protein homologous to the Dps protein of *E. coli* (Frenkiel-Krispin and Minsky, 2002; personal communication, Dr. Vsevolod Popov). SFG rickettsiae usually do not form vacuoles and crystalline structures.

Cultivation Rickettsiae must be cultivated in tissue culture or yolk sac of developing chicken embryos (Cox, 1941). L929 and Vero cells are used most frequently, but a great variety of other cells has been used, including chicken embryo fibroblasts, golden hamster BHK-21 cells, HEL cells, monocytes and polymorphonuclear leukocytes.

In irradiated chicken embryo cells at 34°C, *R. prowazekii* multiplies with a generation time of about 9 h until a very high rickettsial density is reached (Fig. BXII.α.36C). If the inoculum is derived from rickettsiae harvested past their logarithmic growth phase, exponential growth is preceded by a lag phase. The nucleus is not invaded. The rickettsiae are released by the disruption of massively infected cells (Wisseman and Waddell, 1975). Growth in other cell types is qualitatively comparable.

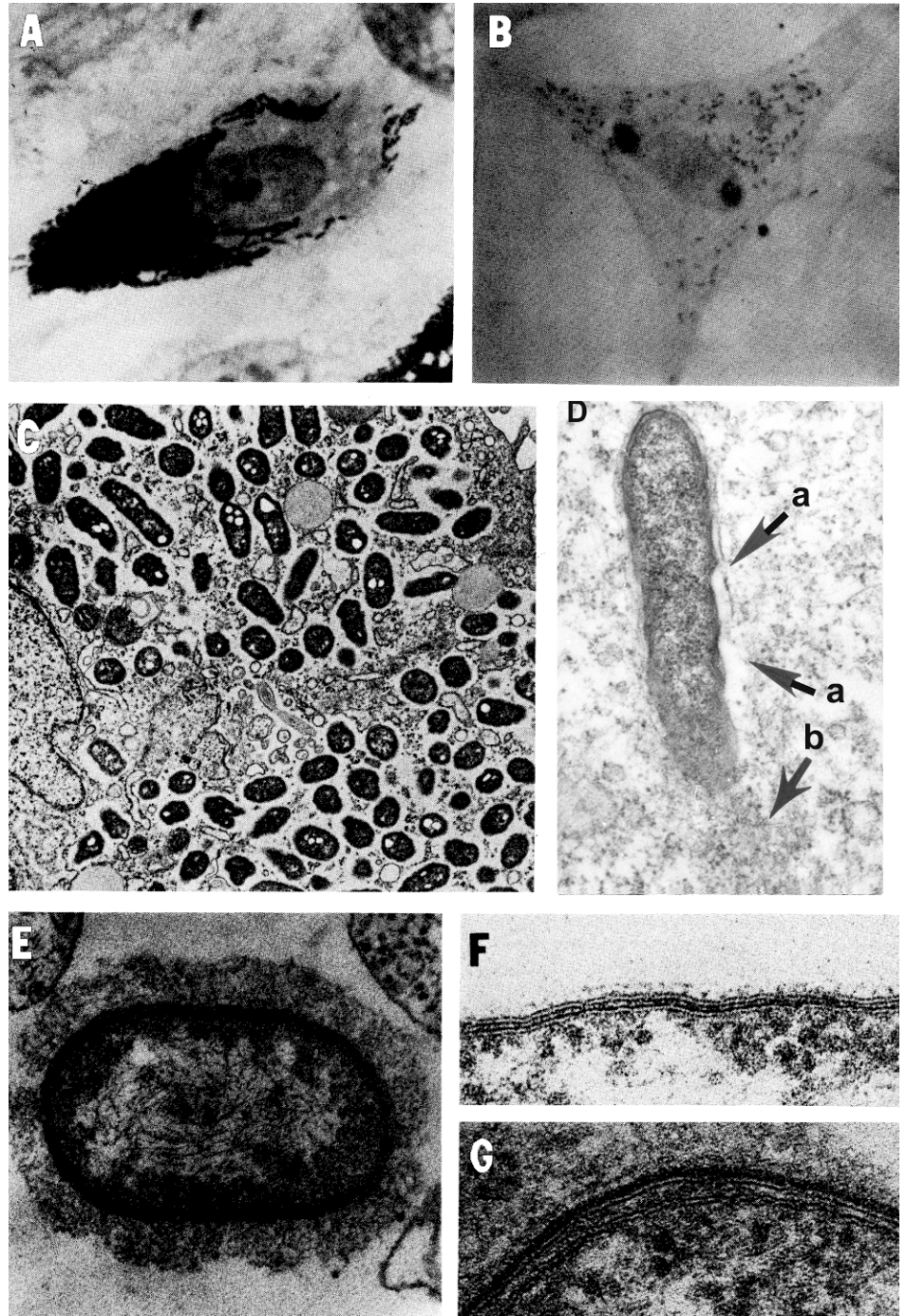
Plaques have been produced on a variety of cell monolayers by procedures similar to, but somewhat more difficult than, those used in virology, since monolayers must be maintained for 5–11 days and the introduction of antibiotics must be carefully avoided (Wike and Burgdorfer, 1972). Plaques appear early and are usually larger for most SFG rickettsiae than with TG rickettsiae. SFG rickettsiae form plaques after 5–8 days with a diameter of 2–3 mm. TG rickettsiae form smaller plaques (1 mm) between 8–10 days. TG rickettsiae produce visible plaques on primary chick embryo fibroblasts and on low-passage mouse embryo fibroblasts but do not form reproducible plaques on continuous cell culture lines. TG rickettsiae may produce visible plaques on continuous cell lines if modified procedures are used. One procedure involves a primary overlay with a medium at pH 6.8, which is followed 2–3 days later with a secondary overlay at neutral pH that contains 1 μg of emetine per ml and 20 μg of NaF per ml. Another procedure involves overlay with a medium containing 50 ng of dextran sulfate per ml (Policastro et al., 1996).

Nutrition and metabolism The nutritional requirements of the rickettsiae, as distinct from those of their host cells, are not known. Rickettsiae grow in heavily irradiated cells (Weiss and Dressler, 1958) that have lost the ability to divide, and they grow in the presence of a low level of cycloheximide that inhibits host protein synthesis. Under these conditions, rickettsiae incorporate exogenous amino acids and adenine, but not thymidine (Weiss et al., 1972). Thus, rickettsial growth occurs independently of host cell protein synthesis, host cell division, and DNA or RNA synthesis. Rickettsiae require a CO₂-enriched atmosphere to grow in chicken embryo cells when an organic buffer is substituted for sodium bicarbonate.

Evolutionarily, rickettsiae are thought to be derived from a free-living bacterial ancestor by the following sequence of events. A free-living aerobic bacterium entered and established an intracellular parasitic relationship with the pre-eucaryote (i.e., an ancestral eucaryote lacking organelles). This made it possible for many chemical substrates to be readily obtained by the bacterium from the host and used without further metabolic modification by the bacterium. The genes of the bacterium involved in metabolic pathways such as glycolysis, fermentation and biosynthesis of small molecules were then redundant with the host cell genes. These redundant genes then had the freedom to mutate, and the mutations might result either in genes with new functions or in pseudogenes. Pseudogenes eventually would be deleted from the genome to make the bacterium more efficient in its use of energy. Gene reduction eventually resulted in a small genome in intracellular bacteria. The loss of genes essential to a free-living mode made the bacterium further dependent on the host cell to supply the substrates, and eventually it lost its ability to live outside the host cell.

Much evidence can be adduced for the above scheme. For instance, no genes required for *de novo* synthesis of nucleotides have been found in the genome of *R. prowazekii*. However, the *R. prowazekii* genome encodes all of the enzymes required for the interconversion of nucleoside monophosphates into all of the other required nucleotides (Andersson et al., 1998). Thus, rickettsiae depend on the monophosphate compounds of the host cell for their nutrition, but they can also utilize their di- and triphosphates. AMP is neither synthesized nor degraded, a situation that seems to be true of the other nucleoside monophosphates as well (Williams and Peterson, 1976). Rickettsiae possibly acquire most amino acids from host cells directly, because only the genes associated with biosynthesis of lysine and serine are found in the genome of *R. prowazekii*. *R. prowazekii*

FIGURE XII.α.36. Interaction with host cells and fine structure of rickettsiae. (A and B) Giménez stained preparations. (C-G) Transmission electron micrographs of ultrathin sections stained with uranyl acetate and lead citrate. A, Human F-1000 fibroblast infected with the Breinl strain of *Rickettsia prowazekii*. A large mass of rickettsiae comprises the entire left portion of the cell with some perinuclear organisms evident. No nuclear involvement is apparent ($\times 1200$). B, Secondary chicken embryo fibroblast infected with the Sheila Smith strain of *Rickettsia rickettsii*. Note the sparse, diffusely distributed cytoplasmic organisms and the two distinct compact masses of the organisms within the nuclear region ($\times 1200$). C, *R. prowazekii*-infected secondary chicken embryo fibroblast late in infection, showing large numbers of free cytoplasmic rickettsiae without vacuolar membrane, most of which contain vacuole-like structures characteristic of typhus organisms in the stationary phase of growth ($\times 13,600$). D, *R. rickettsii* (Sheila Smith strain) in a human endothelial cell (HUVEC) 15 minutes after infection is in the process of escaping from the phagosome. Breaks in the phagosomal membrane (a), and formation of an F-actin tail (b) are apparent ($\times 31,350$). E, *R. prowazekii* (Breinl strain) released from an infected host cell and treated with specific human immune serum to demonstrate the slime layer on the surface of the organism ($\times 67,000$). F, Cell envelope of *R. prowazekii* including microcapsular layer. The envelope of *R. rickettsii* is morphologically similar ($\times 196,000$). G, Cell envelope of *Orientia tsutsugamushi*. Note the thickened outer leaflet of the envelope compared with that of *R. prowazekii* (above) ($\times 171,000$). (From the collection of Charles L. Wisseman, Jr., and David J. Silverman. A, Reproduced with permission from C.L. Wisseman, Jr. and A.D. Waddell, *Infection and Immunity* 11: 1391–1401, 1975, ©American Society for Microbiology; B, Reproduced with permission from C.L. Wisseman, Jr. et al., *Infection and Immunity* 14: 1052–1064, 1976, ©American Society for Microbiology; C, Reproduced with permission from D.J. Silverman et al., *Infection and Immunity* 29: 778–790, 1980, ©American Society for Microbiology; D, Courtesy of M.E. Ereemeeva; E, Reproduced with permission from D.J. Silverman et al., *Infection and Immunity* 22: 233–246, 1978, ©American Society for Microbiology; F and G, Reproduced with permission from D.J. Silverman and C.L. Wisseman, Jr. *Infection and Immunity* 21: 1020–1023, 1978, ©American Society for Microbiology.



requires host cell proline. Maximal rickettsial growth occurs only in host cells with an intracellular proline pool of 1.0 mM or greater (Austin and Winkler, 1988a). *R. prowazekii* also requires host cell serine or glycine for growth (Austin et al., 1987). Interconversion of serine and glycine is catalyzed by a rickettsial serine hydroxymethyltransferase. Rickettsiae have a membrane-bound ATP/ADP translocase that mediates exchange of ATP and ADP. Rickettsiae exchange extracellular ATP for intracellular ADP without a change in the total adenylate pool. The concentration of phosphate acts as a regulatory signal for the rate of

ADP-ATP exchange. The influx of ATP is greatly favored over ADP under conditions of low phosphate in the host cell cytoplasm. Upon transport into the cell, ATP is hydrolyzed by ATPase to generate a membrane potential in the absence of electron transport. Unlike *Chlamydia* cells, which depend totally on the host cell for energy, rickettsiae also synthesize their own ATP (see below). Thus, we do not know to what extent rickettsiae depend on the host cell for ATP. The fact that both *R. prowazekii* and *R. conorii* have five copies of an ATP/ADP translocase gene indicates that a large amount of ATP may be required by rickettsiae from

host cells. The general notion is that rickettsiae acquire host cell ATP early in the infectious cycle via the ATP/ADP translocase and that rickettsiae may synthesize their own ATP to compensate for the depletion of cytosolic ATP late in the infection. The ATP/ADP translocase gene was possibly acquired from the host cell, because it is found only in rickettsiae and chlamydiae (Wolf et al., 1999).

Genes encoding all enzymes of the tricarboxylic acid (TCA) cycle are present in *R. prowazekii*. The primary source of energy is oxidation of glutamate (Bovarnick and Snyder, 1949). Glutamine and pyruvate are also utilized, but to a lesser extent (Weiss, 1973). Glutamine is converted to glutamate by a deamidase. The amino group of the glutamate is transferred to oxaloacetate with the formation of aspartate and α -ketoglutarate by a reversible glutamate-oxaloacetate transaminase. In addition, glutamate is also converted to NH_3 and α -ketoglutarate by a NAD(P)-dependent glutamate dehydrogenase (Weiss, 1973). Glutamate oxidation drives electron transport coupled to oxidative phosphorylation as well as active transport of at least two amino acids, lysine and proline. In *R. prowazekii* the incorporation of P_i into ATP accompanying glutamate oxidation is catalyzed by a membrane-bound ATPase and is sensitive to dicyclohexylcarbodiimide, cyanide, arsenite, and 2,4-dinitrophenol.

Glucose and glucose-6-phosphate are not utilized by rickettsiae. *R. prowazekii* transports uridine 5'-diphosphoglucose (UDPG) but not glucose. *R. prowazekii* takes up glucose phosphates to a much lesser extent than UDPG. *R. prowazekii* does not have hexokinase and phosphoglucomutase, enzymes required for the metabolism of glucose and glucose-6-phosphate (Winkler and Daugherty, 1986). The inability to use glucose as a substrate indicates that rickettsiae may use UDPG as precursors for synthesis of the slime layer, peptidoglycan, and LPS. Rickettsiae must also obtain glycolytic intermediates and products such as acetyl coenzyme A, which are required in the citrate synthase reaction of the TCA cycle. The genes encoding three components (E1-E3) of the pyruvate dehydrogenase complex are found in *R. prowazekii*, indicating that this organism may utilize cytosolic pyruvate. Rickettsiae may use host cell pyruvate by the same mechanism as mitochondria. Pyruvate is imported into mitochondria directly from the cytoplasm, and subsequent conversion into acetyl coenzyme A and CO_2 is catalyzed by pyruvate dehydrogenase. In *R. prowazekii*, pyruvate dehydrogenase activity is dependent on coenzyme A, NAD, and thiamine pyrophosphate and is inhibited by NADH, but not adenylates.

Antigenic structure The major antigens of *Rickettsia* are lipopolysaccharide, lipoprotein, outer membrane proteins, and heat shock proteins. The Weil-Felix reaction has been used as a presumptive diagnostic test for rickettsial diseases. It is based on the cross-reaction of antibodies to rickettsial antigens from primary rickettsial infections with the somatic antigens of three strains: *Proteus vulgaris* strains OX19 and OX2, and *Proteus mirabilis* strain OXK. The rickettsial antigens do not elicit an anamnestic antibody response to these *Proteus* antigens in recrudescence typhus. The antibodies of Japanese spotted fever patients are reactive with the LPS of OX2 as well as OX19. Thus, the cross-reactive antigens between *Rickettsia* and *Proteus* are most likely present in the LPS. The O-polysaccharides of the LPS of typhus rickettsiae are composed of glucose, glucosamine, quinovosamine, and phosphorylated hexosamine, which are also found in the O-polysaccharide of the LPS from *P. vulgaris* OX19 used in the Weil-Felix test. These findings suggest that these O-polysaccharides may represent the antigens common to LPSs from TG rickettsiae and *P. vulgaris* OX19 (Amano et al., 1998).

The antibodies of scrub typhus patients recognize the antigens of OXK only (Amano et al., 1996).

A 17-kDa protein is a genus-common protein of rickettsiae that has been identified in all *Rickettsia* species examined (Anderson, 1990). The sequences of the gene encoding the 17-kDa protein gene are conserved among rickettsial species, indicating the importance of it to the survival of the rickettsiae. The protein is predicted to be a lipoprotein, and part of the protein is surface exposed. Thus, it has been speculated that the 17-kDa protein may play a scaffolding and protective role in the rickettsiae (Anderson, 1990).

All rickettsiae have a 135-kDa outer membrane protein B (OmpB) that has been identified as an S-layer protein of rickettsiae (Ching et al., 1990). OmpB is the most abundant rickettsial surface protein, and it contains species-, group- and genus-specific epitopes (Anacker et al., 1987).

SFG rickettsiae have an additional outer membrane protein, OmpA. *R. felis* and *R. peacockii* have an OmpA that is truncated by the presence of premature stop codons. OmpA contains a hydrophilic region of tandem repeat units, which consists of over 40% of the amino acid sequences (Anderson et al., 1990). The repeat units are not identical and thus are divided into three types, with type I composed of 75 amino acids, type II of 72 amino acids, and type III of 85 amino acids. The type II repeats are less conserved and are further divided into two subtypes (IIa and IIb) (Anderson et al., 1990). The *ompA* sequences up-stream and down-stream of the repeat region are conserved among SFG rickettsial species. Although the sequences of the repeats are conserved among *R. rickettsii*, *R. conorii* and *R. akari*, the number and the order of arrangement of repeat units vary among SFG rickettsiae (Gilmore, 1993). However, the third type of repeat unit found in *R. australis* differs greatly from other SFG rickettsial repeat units. *R. australis* has only type III repeat units, which have only 21% identity to the type I repeat of *R. rickettsii* (Stenos and Walker, 2000). The antigenic diversity of SFG rickettsiae is determined in large part by the number, order, and type of repeat units.

Both OmpA and OmpB have been demonstrated to stimulate protective immunity against rickettsial challenge of vaccinated animals (Sumner et al., 1995; Dasch et al., 1999).

A striking feature of rickettsiae is that all the identified rickettsial outer membrane proteins belong to the autotransporter family. These proteins include OmpA, OmpB, and four ORFs encoding the hypothetical proteins Sca1 (Accession RP018), Sca2 (RP081), Sca3 (RP451), and Sca5 (RP704) (Andersson et al., 1998). Unlike other transport systems, autotransporter proteins are transported across the cytoplasmic or inner membrane and the outer membrane without the assistance of accessory proteins. An autotransporter protein consists of three functional domains: the amino-terminal leader sequence, the secreted mature (α) protein and a carboxy-terminal (β) domain. The signal peptide inserts itself into the inner membrane, directing the export of the precursor molecule by action of the general secretion pathway into the periplasm. The precursor is cleaved by the signal peptidase to release the mature polypeptide into the periplasm. Once in the periplasm, the β -domain of the protein is inserted into the outer membrane to form a β -barrel pore, and the passenger domain is translocated to the cell surface through the pore. The precursor of OmpB is a 168 kDa-protein that is post-translationally processed to the 135-kDa mature OmpB by cleavage of a 32-kDa β -peptide (Hackstadt et al., 1992).

The amino acid sequences of the β -peptides of all five rickettsial autotransporters are highly homologous and share ho-

mology with autotransporters from distantly related bacteria (Henderson et al., 1998). The diversity among the passenger domains that are coupled with the conserved β -domain suggests that the autotransporters are either derived originally from a single gene or resulted from recombination of unrelated proteins with the β -domains (Henderson et al., 1998). Sca 1 and Sca 2 consist of only the β -domain without the passenger peptide. Thus, Sca 1 and Sca 2 may be involved in the transport of other rickettsial proteins. Most of the autotransporter proteins are adhesins or proteases of bacteria, indicating that OmpA, OmpB, Sca3, and Sca5 might be important rickettsial virulence factors.

Drug and antibiotic susceptibility Rickettsiae are naturally resistant to most classes of antimicrobial agents. Contemporary methods determine rickettsial susceptibility to antimicrobial drugs in cell culture. Even the most effective antimicrobial activities against *Rickettsia* are bacteriostatic rather than bactericidal. The antimicrobial drugs that are most effective, i.e., that have the lowest minimal inhibitory concentrations, belong to the following classes: tetracyclines, chloramphenicol, rifampin, fluoroquinolones (pefloxacin, ofloxacin, ciprofloxacin) and some, but not all, macrolides (josamycin, azithromycin, clarithromycin). Doxycycline is the drug of choice in most clinical settings, except for patients who are pregnant or hypersensitive to tetracyclines (Raoult et al., 1990; Walker and Sexton, 1999). There has been long empiric experience with the use of chloramphenicol for the successful treatment of rickettsioses, but the outcome is less satisfactory for Rocky Mountain spotted fever than treatment with doxycycline (Holman et al., 2001). Josamycin has been shown to be an effective treatment of boutonneuse fever during pregnancy. Ciprofloxacin, ofloxacin, pefloxacin, clarithromycin, and azithromycin have been reported to ameliorate the course of boutonneuse fever (Bella et al., 1990; Cascio et al., 2001). The results of treatment with rifampin have been less conclusive, and *R. massiliae*, *R. aeschlimannii*, *R. montanensis*, and *R. rhipicephali* are relatively resistant to rifampin. *Rickettsia prowazekii* is susceptible to erythromycin *in vitro*; however, rapid selection of antimicrobial resistant mutants occurs. *Rickettsia* species are resistant to aminoglycosides and to penicillin and other β -lactam antimicrobial agents.

Sulfonamides are not only ineffective, they actually exacerbate rickettsial infections. In contrast, an analog of sulfonamide, *p*-aminobenzoic acid, is rickettsiostatic and was used successfully to treat Rocky Mountain spotted fever in the 1940s prior to the availability of broad spectrum antibiotics.

Pathogenicity A number of *Rickettsia* species cause severe disease in humans. Before the advent of the broad-spectrum antibiotics, epidemic typhus and Rocky Mountain spotted fever—caused by *R. prowazekii* and *R. rickettsii*, respectively—had a very high case fatality rate. Epidemic typhus has changed the course of history on many occasions (Zinsser, 1935). Rocky Mountain spotted fever and epidemic typhus have exacted a heavy toll on their early investigators.

Other named rickettsiae and their diseases include *R. typhi* (murine typhus), *R. conorii* (boutonneuse fever, Mediterranean spotted fever, Astrakhan fever, Israeli spotted fever), *R. sibirica* (North Asian tick typhus), *R. australis* (Queensland tick typhus), *R. akari* (rickettsialpox), *R. japonica* (Japanese spotted fever), *R. africae* (African tick bite fever), *R. honei* (Flinders Island spotted fever), and *R. felis* (flea-borne spotted fever).

Rickettsiae are inoculated into the skin in the saliva during feeding by an infected tick, mite, or flea or by scratching of rickettsia-laden feces deposited by an infected louse or flea. Rick-

ettsiae are distributed throughout the body via the bloodstream where they enter their principal target, endothelial cells.

In the interaction of rickettsiae with host cells, the entry process involves three steps: attachment, internalization and escape from the phagosome. Rickettsiae adhere to the host cell by means of a rickettsial adhesin and a host cell receptor. Experimental evidence indicates that a ligand and receptor interaction mediate rickettsial attachment. Adsorption of *R. prowazekii* to L-cells and human endothelial cells can be saturated and is dependent on time and multiplicity of infection (Walker and Winkler, 1978; Walker, 1984). Pure *R. prowazekii* is more efficient than a crude suspension in entry into chicken embryo cells (Wisseman et al., 1976). The difference is due to competition between rickettsial receptors on host cell membrane fragments and on intact host cells. The receptors on host cells have yet to be identified, although experimental evidence suggests the host receptor for rickettsiae contains cholesterol as a binding moiety. *R. prowazekii* binds to the cholesterol-containing fraction of chloroform-methanol extracts of erythrocyte membranes (Ramm and Winkler, 1976). Digitonin and amphotericin B, which bind to cholesterol, inhibit the adsorption of *R. prowazekii* to erythrocyte ghosts and reduce plaque formation by *R. rickettsii* in chicken embryo cell monolayers (Walker et al., 1983). However, there is no effect of these agents on the interaction of *R. prowazekii* and L929 cells (Austin and Winkler, 1988b).

OmpA has been implicated as an adhesin of *R. rickettsii* because monoclonal antibodies to OmpA block *R. rickettsii* attachment (Li and Walker, 1998). There is evidence that both OmpA and OmpB are adhesins of *R. japonica* (Uchiyama, 1999).

Rickettsiae enter the host cell via induced phagocytosis that requires undefined rickettsial activity and host cell cytoskeletal rearrangements. Adsorption and internalization require metabolically competent rickettsiae and active participation of the host cell. Inactivation of *R. prowazekii* results in decreased adsorption to L929 cells and endothelial cells (Walker, 1984). *R. prowazekii* adheres to, but is unable to enter, cells treated with a cytoskeletal actin polymerization inhibitor such as cytochalasin B (Walker and Winkler, 1978). Owing to rickettsial infection of nonphagocytic cells, the internalization process is usually called invasion.

Once phagocytosed by the host cell, rickettsiae rapidly escape from the phagosome prior to its fusion with a lysosome (Fig. BXII.α.36D). A rickettsial enzyme is hypothesized to lyse the phagosome membrane to release rickettsiae. The enzyme is most likely a phospholipase because the incubation of *R. prowazekii* with erythrocytes results in hemolysis and formation of free fatty acids and lysophosphatides (Winkler and Miller, 1981; Walker et al., 2001). The phospholipase is of rickettsial origin rather than host cell origin (Winkler, 1990). The nature of the rickettsial phospholipase has not been determined directly. Although phospholipase A2 (PLA2) has been suggested as the rickettsial enzyme that lyses the phagosomal membrane, rickettsial genome sequencing has not revealed a gene encoding a PLA2. A rickettsial protein (Accession RP534) has a calcium-independent PLA2 motif (Yu et al., 2000b), which is homologous to the gene for a cytotoxin (ExoU) of *Pseudomonas aeruginosa* (Vallis et al., 1999). Within the cytosol, rickettsiae acquire nutrients, ATP, amino acids, and nucleic acid precursors from the host by active transport mechanisms and replicate slowly (Winkler, 1990).

SFG rickettsiae stimulate actin-based mobility, resulting in intercellular spread. TG rickettsiae accumulate to massive quantities intracellularly until the cell bursts, releasing the rickettsiae. Observations in cell culture systems suggest that the mechanisms

of intracellular movement and destruction of the host cell differ among SFG and TG rickettsiae (Silverman and Wiseman, 1979; Silverman et al., 1980). TG rickettsiae are released from host cells by lysis of the cells. After infection with *R. prowazekii* or *R. typhi*, the rickettsiae continue to multiply until the cell is packed with organisms and then bursts. Cell death possibly results from apoptosis or from membranolytic activity that previously has been hypothesized to be phospholipase A2 (Winkler and Miller, 1982; Walker et al., 1984; Winkler and Daugherty, 1989; Silverman et al., 1992; Manor et al., 1994; Winkler et al., 1994; Ojcius et al., 1995). Before lysis, TG rickettsia-infected host cells have a normal ultrastructural appearance. SFG rickettsiae seldom accumulate in large numbers and do not burst the host cells. They escape from the cell by stimulating polymerization of host cell-derived F-actin tails (Fig. BXII.α.37), which propel them through the cytoplasm and into filopodia, from the tip of which they emerge (Schaechter et al., 1957; Teyssie et al., 1992; Heinzen et al., 1993, 1999). The rickettsial protein responsible for the actin-based movement of SFG rickettsiae has yet to be identified. The genome sequence of *R. conorii* has not revealed a rickettsial protein homologous to ActA or Ics, the proteins responsible for actin polymerization by *Listeria monocytogenes* and *Shigella flexneri*, respectively; however, a hypothetical protein of 520 residues (RC0909) exhibits an overall organization similar to that of ActA. Both proteins are highly charged at the N-terminus and have a central proline-rich region. RC0909 has a weak similarity to the WASP (Wiskott-Aldrich Syndrome Protein) homology domain 2, which regulates the formation of the actin filaments (Ogata et al., 2001).

Rickettsia rickettsii stimulates the production of reactive oxygen species by infected endothelial cells, resulting in oxidative stress and lipid peroxidation-mediated injury to host cell membranes (Walker et al., 1983, 1984; Silverman and Santucci, 1988; 1990;

Eremeeva et al., 2001; Walker et al., 2001). *R. rickettsii* also directly stimulates activation of transcription factor NF- κ B by a proteasome-independent mechanism. The consequences include inhibition of endothelial cell apoptosis as well as proinflammatory effects (Sporn et al., 1997; Sahni et al., 1998). The key pathophysiological effect of disseminated rickettsial infection of endothelium is increased vascular permeability—a life-threatening event in the brain and lungs in Rocky Mountain spotted fever and epidemic typhus and in a smaller proportion of patients with boutonneuse fever and murine typhus. A hallmark of most rickettsioses is a rash that represents multiple focal networks of rickettsia-infected microvascular endothelium in the skin. Except in Rocky Mountain spotted fever and epidemic and murine typhus, another characteristic visible sign of rickettsiosis is an eschar—the site of cutaneous necrosis where the tick or mite inoculated the rickettsiae.

Antirickettsial immune mechanisms are dominated by T-lymphocyte-mediated cellular immunity. Cytokines, particularly gamma interferon and tumor necrosis factor- α , secreted by T-lymphocytes, macrophages, and natural killer cells, activate infected endothelial cells and other cells to kill intracellular rickettsiae by nitric oxide-dependent mechanisms and other mechanisms. Cytotoxic CD8 T-lymphocytes are critical effectors of the clearance of rickettsiae. Antibodies directed against OmpA and OmpB, but not lipopolysaccharide, also contribute to protective immunity. The potential pathologic effects of cytokines and cytotoxic T-lymphocyte activity have not been determined.

Ecology A consistent characteristic of *Rickettsia* species is their residence in an arthropod host as at least a part of their ecological niche. Transovarian maintenance from one generation of tick, mite, or flea to the next via infected ova that hatch into infected larvae is a factor in the maintenance in nature of all SFG rickettsiae (e.g., *R. akari*, *R. australis*, *R. conorii*, *R. felis*, *R. honei*, *R. peacockii*, *R. rickettsii*, and *R. sibirica*) as well as *R. typhi*. For some *Rickettsia* species, such as *R. peacockii*, transovarian transmission appears to be the only mechanism of survival (Niebylski et al., 1996, 1997b). For others, such as *R. rickettsii*, the organism exerts a pathologic effect on the tick that would eventually result in extinction of the rickettsia (Niebylski et al., 1999). Thus, it appears that its virulence comprises an additional survival strategy, the ability to invade and grow in a vertebrate host (e.g., cotton rats) to a sufficient level and duration of rickettsemia to establish infection in uninfected feeding larval and nymphal ticks, with the subsequent transovarian maintenance of the rickettsiae. In this way the death of infected ticks that is brought about by the pathogenic effects of their rickettsial infection is balanced by replacement by new infected tick lines. Ticks infected with highly virulent rickettsiae (e.g., *R. rickettsii*) are infected by fewer rickettsiae than ticks infected with rickettsiae of no apparent pathogenicity (e.g., *R. montanensis*). For *R. typhi*, transovarian maintenance is less important than horizontal spread via rickettsemic *Rattus*.

In the human-body louse (*Pediculus humanus corporis*) cycle of *R. prowazekii*, the lice do not transmit the rickettsiae vertically to the next generation. Indeed, *R. prowazekii* kills 100% of infected *P. humanus corporis* lice. However, the likely natural cycle of *R. prowazekii* in the flying squirrel (*Glaucomys volans*) involves its own flea (*Orchopeas howardii*) and louse (*Neohematopinus sciuropteri*) without severe pathologic effects on the vertebrate and invertebrate hosts (Bozeman et al., 1975, 1981). Epidemics of louse-borne typhus depend upon the establishment of latent human infection with *R. prowazekii*. Years later, recrudescence of the infection occurs, accompanied by rickettsemia. Human body lice

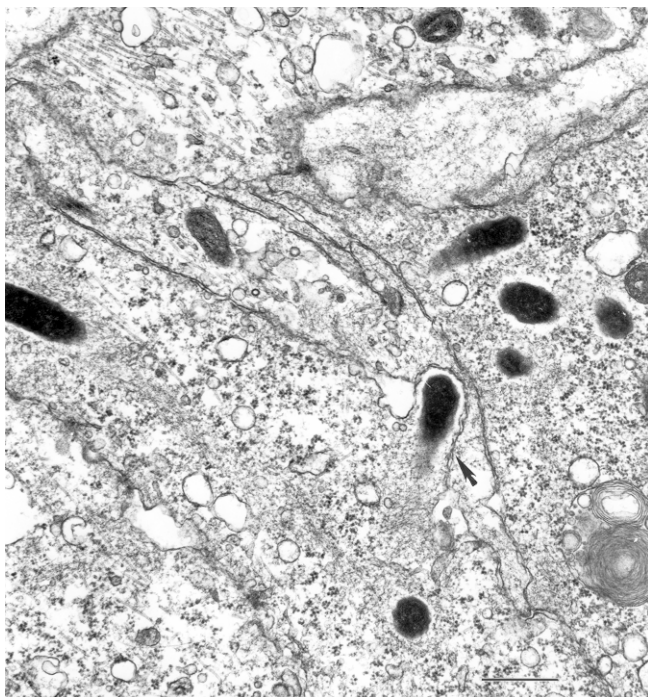


FIGURE BXII.α.37. Electron photomicrograph of *Rickettsia conorii*-infected Vero cells. A rickettsial cell with an actin tail protrudes into an adjacent cell (arrowhead). Bar represents 1 μ m. Photomicrograph courtesy of Vsevolod Popov.

acquire the rickettsiae by feeding upon the patient's infected blood and then move to nonimmune persons. It is postulated that this human-louse cycle was first established in North America after accidental human infection originating from contact with infected flying squirrel ectoparasites in the pre-Columbian era. Human body lice were present in the New World at that time.

Knowledge of rickettsial ecology is preponderantly based on investigations of medical epidemiology and hematophagous arthropods. The discovery of rickettsial strains closely related to *R. bellii* in herbivorous insects and in association with plant pathology (papaya bunchy top disease) indicates that our view of the diversity and evolutionary origin of *Rickettsia* is likely overly anthropocentric.

ENRICHMENT AND ISOLATION PROCEDURES

Isolation of rickettsiae from ecologic survey specimens or from clinical material requires strict adherence to safety precautions. A biological safety level 3 facility and an approved safety cabinet in which negative air pressure is maintained are recommended.

Rickettsiae are most commonly isolated in cell culture, although historically the isolation of rickettsiae employed inoculation of adult male guinea pigs or embryonated chicken eggs (Walker, 1996). Vero, L-929, HEL, and MRC5 are cell lines commonly used for isolation of rickettsiae. Heparin-anticoagulated plasma or buffy coat ideally is collected before the patient is given antirickettsial treatment. Isolation of rickettsiae is enhanced by centrifugation of the inoculum onto monolayers in shell vials (La Scola and Raoult, 1996a). Rickettsiae are detected by examining monolayers stained by Giemsa, Giménez, or immunofluorescence methods with 82% of positive samples identified after 48 hours incubation. Rickettsiae are susceptible to some classes of antibiotics. Thus, the culture media ideally should not contain antibiotics although penicillin, streptomycin, and sulfonamides have been used to suppress the growth of bacteria from contaminated specimens.

If the samples are contaminated with bacteria or if cell culture is not available, rickettsiae can be also isolated using animals. The guinea pig is the animal of choice for rickettsiae of the typhus and spotted fever groups. Burgdorfer et al. (1975) found the meadow vole (*Microtus pennsylvanicus*) to be particularly susceptible and valuable for the isolation of spotted fever rickettsiae. The mouse is the animal of choice for *R. australis* and *R. akari*. Rickettsial species vary considerably in virulence for the guinea pig and mouse (Table BXII.α.31). Spleen and other organs are collected from guinea pigs during the 2nd and 3rd day of fever or from moribund mice and are passed into cell culture or embryonated eggs. When a rickettsial species of low virulence, such as *R. montanensis* is expected, the spleen is passed on the 10th–12th day after injection, even in the absence of fever or other signs of illness.

Chicken embryos are generally used for the production of stocks of rickettsiae isolated in laboratory animals. Embryonated eggs can also be used for primary isolation if the inoculum is free from adventitious agents (blood or ticks whose exterior surfaces have been decontaminated by immersion in Merthiolate and by repeated washing). Chicken embryos must be obtained from flocks maintained on a rigorous antibiotic-free diet. Yolk sac inoculation during the 5th–7th d of embryo development is the only satisfactory route for propagation of rickettsiae in eggs (Cox, 1941). The optimal conditions for cultivation and harvest vary with the rickettsial group (Table BXII.α.31).

Rickettsiae have also been isolated from the hemolymph of

ticks by the shell vial centrifugation-enhanced method of cell culture. A drop of hemolymph is obtained by amputating the distal portion of one or more legs and is inoculated into a shell vial containing a L929 cell monolayer. The vials are centrifuged at $700 \times g$ for 1 h to increase rickettsial attachment and entry into the cells.

Purification of rickettsiae is accomplished by the separation of rickettsiae from host cell components by differential centrifugation or density gradient centrifugation.

Release of rickettsiae from host cells is achieved as follows. If rickettsiae are grown in yolk sacs, the yolk sacs should be ground using a tissue homogenizer and diluted in 10 volumes of sucrose phosphate glutamate (SPG) buffer (0.22 M sucrose, 0.01 M potassium phosphate and 0.005 M potassium glutamate [pH 7.0]) (Bovarnick et al., 1950). If cell culture is used to propagate rickettsiae, the cells should be harvested in SPG buffer and sonicated for 10-sec periods several times on ice until most of cells are broken.

To remove large particles of debris, the rickettsial suspension is centrifuged at $200 \times g$ for 10 min. The supernatant is then centrifuged at $10,000 \times g$ for 20 min to pellet the rickettsiae. The aqueous phase (and fat layer if yolk sacs are involved) is discarded, and the pellet is resuspended in SPG buffer in an appropriate volume for the Renografin centrifugation.

Renografin is the common gradient material used for purification of rickettsiae by density gradient centrifugation (Weiss et al., 1975). The gradient can be either linear or discontinuous. A linear gradient is made using two concentrations of Renografin, 32% and 42%, by a gradient mixer. A discontinuous gradient consists of Renografin concentrations of 32, 36, and 42%. A discontinuous gradient may be made by adding the solutions in ascending or descending order. If the solution is added in ascending order, the 42% solution is added to the bottom of the centrifuge, then the 36% solution is added slowly on top of the 42% solution using a pipette or a syringe along the edge of the tube. The tip of the pipette or the syringe should just touch the surface of the 42% solution to avoid disturbing the solution. Finally, the 32% solution is added on top of the 36% solution by same method. If the solution is added in descending order, the 32% solution is added into the bottom of the centrifuge tube, then the 36% solution is added slowly by inserting the tip of the pipette or syringe to the bottom of the tube. Finally, the 42% solution is added slowly by inserting the tip of the pipette or syringe to the bottom of the tube. A rickettsial suspension prepared by differential centrifugation is added on top of the Renografin solution. Five ml of rickettsial suspension may be added to a 30 ml Renografin gradient column. The rickettsiae are centrifuged at $87,275 \times g$ for 60 min. Two bands (the heavy and light bands) are usually visible at the interfaces between the 32% and 36% Renografin layers and between the 36% and 42% Renografin layers. The bands are collected by aspiration using a pipette or a syringe inserted into the middle of a band or by puncture of the centrifuge tube beneath the band. The suspension is diluted using SPG to two volumes minimum and mixed well by inverting the tube. The suspension is centrifuged at $11,400 \times g$ for 20 min. The pellet contains the purified rickettsiae, which are then suspended in SPG buffer for storage.

The organisms in the heavy band are defective in their infective and metabolic activities, as compared to organisms from the light band. The greater density of heavy-banding organisms is due to their lack of an intact permeability barrier to Renografin. The proportion of heavy-banding organisms in a rickett-

TABLE BXII.α.31. Differentiation of the species of the family *Rickettsiaceae*^a

Species	Geographic distribution	Arthropod host	Intracellular location	Peak titer in chicken embryo occurs	Optimal temperature for growth in chicken embryo	Hemolytic activity	Susceptibility to infection	OmpA	LPS ^b
<i>Typhus group</i>									
<i>R. prowazekii</i>	Worldwide	Human louse, flying squirrel flea and louse	Cytoplasm	Prior to death	35°C	+	Guinea pig	—	T
<i>R. typhi</i>	Worldwide	Flea	Cytoplasm	Prior to death	35°C	+	Guinea pig, mouse	—	T
<i>Spotted fever group</i>									
<i>R. aeschlimannii</i>	Africa	Tick	Cytoplasm ^c	24–72 h after death	32–34°C	—		+	S
<i>R. africae</i>	Africa, Caribbean	Tick	Cytoplasm ^c	24–72 h after death	32–34°C	—	Guinea pig, mouse	+	S
<i>R. akari</i>	Worldwide	Mite	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Mouse	+	S
<i>R. australis</i>	Australia	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Guinea pig	+	S
<i>R. conorii</i>	Eurasia, Africa	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—		+	S
<i>R. felis</i>	Americas, Europe	Flea	Cytoplasm ^c					+	S
<i>R. helvetica</i>	Europe, Asia	Tick	Cytoplasm ^c					+	S
<i>R. honei</i>	Australia, Asia	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—		+	S
<i>R. japonica</i>	East Asia	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—		+	S
<i>R. massiliae</i>	Europe	Tick	Cytoplasm ^c					+	S
<i>R. montanensis</i>	America	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—		+	S
<i>R. parkeri</i>	America	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Guinea pig	+	S
<i>R. peacockii</i>	America	Tick	Cytoplasm					— ^c	S
<i>R. rhipicephali</i>	Americas	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Guinea pig	+	S
<i>R. rickettsii</i>	Asia	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Guinea pig	+	S
<i>R. sibirica</i>	Europe	Tick	Cytoplasm, nucleus	24–72 h after death	32–34°C	—	Guinea pig	+	S
<i>R. slovaca</i>									
<i>Ancestral group</i>									
<i>R. bellii</i>	America	Tick	Cytoplasm, nucleus	Prior to death	32–34°C	—		+	B
<i>R. canadensis</i>	America	Tick	Cytoplasm, nucleus	Prior to death	35°C	+		+	T
<i>Scrub typhus group</i>									
<i>O. tsutsugamushi</i>	Asia, Australia	Mite	Cytoplasm, nucleus	Prior to death	35°C	—	Mouse	—	—

^aFor symbols see standard definitions.^bLPS type: T typhus type, S spotted fever type, B = *R. bellii* type.^cNucleus location was not reported.^d*R. felis* has a truncated ompA.^e*R. peacockii* has an ompA pseudogene.

sial suspension is influenced by the growth phase when they are harvested from infected yolk sacs, as well as by the conditions and media to which they subsequently are exposed (Hanson et al., 1981).

MAINTENANCE PROCEDURES

Rickettsial stocks are usually prepared from infected cell culture or yolk sacs. Infected cells should be harvested before all the cells have detached from the flask. Portions of the infected cells are stored as stock. Infected yolk sacs are ground and diluted to 10 volumes in SPG buffer. Stocks of rickettsiae have been maintained as crude yolk sac suspensions for decades at -70°C . When diluted or separated from host constituents, viability declines at rates that depend on the degree of purification and on the choice of suspending fluid. The diluent most frequently used as the maintenance medium is SPG buffer. Stability is further enhanced by the addition of Mg^{2+} and a substance such as brain-heart infusion broth (BHI), bovine serum albumin, or Renografin. Storage in liquid nitrogen is recommended for specimens of unusual value.

Rickettsiae lose viability when they are repeatedly frozen and thawed, maintained at 4°C for a few days, or kept at room tem-

perature for several hours. However, they may be quite stable in desiccated arthropods or louse feces, which occasionally have been a source of unexpected infection (see Smadel, 1965; Snyder, 1965; Woodward and Jackson, 1965).

DIFFERENTIATION OF THE GENUS *RICKETTSIA* FROM OTHER GENERA

Rickettsiae can be easily differentiated from free-living bacteria by their obligately intracellular growth feature. The genus *Rickettsia* can be differentiated from *Anaplasma*, *Ehrlichia*, and *Coxiella* by its intracellular location. Rickettsiae grow free in the cytoplasm or nucleus as compared to *Anaplasma*, *Ehrlichia*, and *Coxiella*, which grow in the cytoplasm within a vacuole. The characteristics for differentiation between *Rickettsia* and *Orientia* are summarized in Table BXII.α.32.

ACKNOWLEDGMENTS

We are indebted to Drs. David J. Silverman Department of Microbiology, School of Medicine, University of Maryland, Baltimore, Vsevolod Popov, Department of Pathology, University of Texas Medical Branch, Galveston, Akira Tamura and Hiroshi Urakami, Department of Microbiology, Niigata College of Pharmacy, Niigata, for providing the illustrations.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RICKETTSIA*

The differential characteristics of the three groups of *Rickettsia* and some of the species characteristics are presented in Table BXII.α.31. Although most species display some obvious differ-

ential characteristics, final identification requires genetic and antigenic analysis.

List of species of the genus *Rickettsia*

1. ***Rickettsia prowazekii*** da Rocha-Lima 1916, 567^{AL} (Nom. Cons. Opin. 19, Jud. Comm. 1958, 158.) *pro.wa.ze'ki.i*. M.L. gen. n. *prowazekii* of Prowazek; named after Stanislav von Prowazek, an early investigator of the etiology of typhus who died of typhus contracted in the course of his studies.

The description of the genus and, in particular, of the typhus group within the genus is based largely on studies of this species. Although cells are generally small, variation in size and morphology is most pronounced in this species. Unusually long cells (4 μm , single or in short chains) and cells with prominent vacuoles appear with moderate frequency in stationary cultures.

Highly infectious for chicken embryos, which die within 4–13 days after inoculation, depending on the quantity of infectious organisms in the inoculum. Optimal yields of rickettsiae are obtained by inoculating the embryos with small inocula (10 viable rickettsiae per egg) and harvesting the yolk sacs 10–14 days later, just before the embryos die. Highly infectious also for monolayers of chick embryo fi-

broblasts, L-929 cells, and Vero cells. Plaques are usually small (0.5–1.5 mm in diameter) and turbid (see Table BXII.α.31). The virulent Breinl strain, but not the avirulent E strain, multiplies to a moderate extent in human macrophage cultures (Gambrill and Wisseman, 1973).

R. prowazekii is the etiologic agent of epidemic typhus fever, which is acquired through contact with lice. It is also the etiologic agent of recrudescent typhus (called Brill-Zinsser disease) and occasionally of sporadic typhus in individuals who have been in contact with flying squirrels or their ectoparasites (McDade et al., 1980; Duma et al., 1981). The most effective arthropod vector of epidemic typhus is the human body louse, *Pediculus humanus corporis*, possibly because it takes frequent large blood meals and because it tends to desert febrile hosts to seek new ones. The head louse is equally susceptible to infection but has not been implicated in typhus fever transmission, possibly because it imbibes only very small amounts of blood (Murray and Torrey, 1975). When the rickettsiae are ingested in an infectious blood meal, they enter in the midgut epithelial cells of the louse and undergo massive multiplication. The rickettsiae grow profusely in the cells of the gut epithelium of the louse, even when the ingested human blood contains high levels of antibodies (Wisseman and Waddell, 1975). Heavily infected epithelial cells are released into the lumen, are discharged with the feces of the louse, and are the source of human infection when the louse feces are inoculated into the skin by scratching. Lice invariably succumb to infection, usually within 1–2 weeks, rarely surviving longer than 3 weeks. When louse infestation is very heavy or when large volumes of rickettsial suspensions are processed in the laboratory, infection may occur by inhalation of aerosols.

TABLE BXII.α.32. Differentiation of the genera of the family *Rickettsiaceae*^a

	<i>Rickettsia</i>	<i>Orientia</i>
<i>Cell wall components</i>		
LPS	+	—
Peptidoglycan	+	—
OmpB	+	—
17 kDa predicted lipoprotein	+	—
56 kDa protein	—	+
Mol% G + C of the DNA	29–33	28.1–30.5
Electron lucent zone	+	—

^aFor symbols see standard definitions.

The guinea pig is highly susceptible to infection, but develops a mild disease, usually manifested only as fever lasting approximately a week. The cotton rat, *Sigmodon hispidus*, is also highly susceptible to infection, but signs of disease and death are produced only by doses in excess of 3×10^5 viable rickettsiae. Inapparent infection elicits solid immunity to the homologous species and to *R. typhi*. True infection does not occur in the mouse, but acute toxic death is produced by the intravenous injection of at least 10^6 viable rickettsiae.

Of the laboratory-induced variants of *R. prowazekii*, the most notable is strain E, isolated from a typhus patient in Madrid in 1941 and passed in rapid succession in eggs 255 times. This strain has limited virulence for the guinea pig and low virulence for man. It has been used as a living vaccine (Fox, 1956). Other laboratory-induced variants have been obtained from strain E. They include strains unaffected by erythromycin and strains of increased resistance to *p*-aminobenzoic (PABA) and to chloramphenicol (Weiss, 1960; Weiss and Dressler, 1962). Strain E easily reverts to virulence during passage in guinea pigs. The mechanisms of attenuation and reversion to virulence are not known, although virulence is associated with differences in methylation of lysine residues of OmpB (Ching et al., 1993).

Strains isolated from various geographic locations are remarkably similar to each other in biological properties. Differences in % DNA-DNA hybridization were negligible (range 92–100; mean = 97) among strains isolated in Poland, Spain, and Burundi from human sources and from flying squirrels from the United States (Myers and Wiseman, 1980). Minor differences in protein migration patterns obtained by isoelectric focusing in polyacrylamide gels can distinguish strains derived from Eastern Europe, Spain or Africa, or from American flying squirrels. Another minor difference between the attenuated E strain and the virulent strains in protein migration patterns has been noted (Dasch et al., 1978). The genome size of *R. prowazekii* is 1,111,523 bp (Andersson et al., 1998). No resident plasmid or bacteriophage has been identified in *R. prowazekii*. The *R. prowazekii* genome has 834 open reading frames.

The mol% G + C of the DNA is: 29.0 (T_m).

Type strain: Breinl strain, ATCC VR-142.

GenBank accession number (16S rRNA): M21789.

2. **Rickettsia aeschlimannii** Beati, Meskini, Thiers and Raoult 1997, 553^{VP}

ae.schli.man'ni.i. L. gen. n. *aeschlimannii* of Aeschlimann, named after Andre Aeschlimann, a Swiss zoologist.

The organisms are 0.7–1.1 μm in length, with a mean diameter of 0.3 μm . *R. aeschlimannii* grows in Vero, L929, and HEL cell lines. Mouse antisera against the organism show cross-reactivity with antigens of other SFG rickettsiae. 16S rRNA gene sequence analysis can distinguish *R. aeschlimannii* from other SFG rickettsiae. The most closely related species are *R. massiliae* and *R. rhipicephali*. The sole available isolate was obtained from *Hyalomma marginatum* collected in Morocco (Beati et al., 1997). Two human patients have been reported as infected with *R. aeschlimannii* (Raoult et al., 2002; Pretorius and Birtles, 2002).

The mol% G + C of the DNA is: not determined.

Type strain: MC16 (Reference Center for Rickettsioses, Marseille, France).

3. **Rickettsia africae** Kelly, Beati, Mason, Matthewman, Roux and Raoult 1996, 612^{VP}

a'fri.cae. L. gen. n. *africae* pertaining to Africa, the continent where the organism was isolated.

The dimensions of the organisms are $0.3\text{--}0.5 \times 0.9\text{--}1.6$ μm . Growth occurs in the yolk sacs of chicken embryos, L929, Vero, and human embryonic lung fibroblast cells. Plaque formation does not occur in infected Vero cells. Electron microscopy shows that the organisms grow free in the cytoplasm with an outer electron lucent zone and a trilaminar cell wall. The organism can be differentiated from closely related rickettsiae such as *R. parkeri*, *R. sibirica*, and *R. rickettsii* by pulsed field gel electrophoresis after digestion of the DNA with *EagI*, *SamI*, and *BssHII*. The 16S rRNA gene sequences of *R. africae* have 99.9% similarity to those of *R. conorii*, and 99.7% similarity to those of *R. sibirica*. The genome size of *R. africae* is 1.248 kb.

The reservoirs of *R. africae* are *Amblyomma hebraeum* and *A. variegatum*. Rickettsiae can be maintained transstadially and transovarially in *A. hebraeum*; 72% of *A. hebraeum* in Zimbabwe carry these rickettsiae (Kelly et al., 1996).

R. africae causes African tick bite fever. The disease has been reported from sub-Saharan Africa where boutonneuse fever is also endemic. African tick bite fever is a mild disease, whereas boutonneuse fever may be more severe. The skin rash may be sparse, vesicular, or absent. Many patients have multiple eschars and associated regional lymphadenopathy (Kelly et al., 1996).

The mol% G + C of the DNA is: not determined.

Type strain: Z9-Hu (Reference Center for Rickettsioses, Marseille, France).

4. **Rickettsia akari** Huebner, Jellison and Pomerantz 1946, 1682^{AL}

a.ka'ri. Gr. neut. n. *akari* a mite.

Among SFG rickettsiae, *R. akari* is relatively distantly related to *R. rickettsii*. It grows somewhat more profusely in the yolk sac of chicken embryos than does *R. rickettsii* (Shepard and Luncford, 1976) and is somewhat more cytotoxic when grown in cell cultures (Weiss et al., 1972; Kokorin et al., 1978). The mouse, however, is highly susceptible to infection and is the animal of choice for isolation. Differences in virulence between strains of this species and differences in susceptibility between inbred strains of mice have been demonstrated (Anderson and Osterman, 1980). The moderate virulence for the guinea pig is about the same as that of *R. conorii*.

In man, *R. akari* causes rickettsialpox. This disease is characterized by a generalized, typically vesicular rash, fever, malaise, and lymphadenitis, which resolve in about a week. The disease in man was first observed in 1946 in New York City (Huebner et al., 1946). It has been reported mainly from urban areas along the Atlantic coast of the United States, in the Crimea and southern Ukraine and Croatia (Radulovic et al., 1996). In the mid-1940s, about 180 cases were reported annually in the United States, but only sporadic cases have been recognized in recent years (Wong et al., 1979).

This species is readily distinguished from the other members of the spotted fever group by DNA sequence analysis and immunologic evaluation. The main vector is the mite, *Liponyssoides sanguineus*, which maintains the rickettsia transovarially. The nymph and adult stages of the mite feed on the house mouse, *Mus musculus*, but they may attack other animals and man. This *Rickettsia* has also been isolated

from rats and from a wild Korean rodent, *Microtus fortis pelliceus* (Jackson et al., 1957).

The genome has a size of 1,231,204 bp and contains 1677 open reading frames and 33 tRNA genes (Eremeeva et al., 2002). Differences in electrophoretic migration patterns of the solubilized proteins between *R. akari* and the other species of the spotted fever group are easily visualized (Obijeski et al., 1974; Pedersen and Walters, 1978).

The mol% G + C of the DNA is: 32.3 (determined from the genome sequence).

Type strain: Kaplan, MK, ATCC VR-148.

This strain was isolated from the blood of a patient in New York City (Huebner et al., 1946). Strain 29 isolated from a mouse by Fuller et al. (1951) and a strain isolated from a mite in the U.S.S.R. (received from Zhdanov [Zhdanov and Korenblit, 1950]) are frequently used as reference strains.

5. ***Rickettsia australis*** Philip 1950, 786^{AL}
aus.tra'lis. L. fem. adj. *australis* southern.

R. australis is a member of a clade that also includes *R. akari* and *R. felis* and is somewhat separate from the core of the SFG2. *R. australis* is not highly virulent for the guinea pig and produces a relatively mild disease in humans. It is highly virulent for the newborn mouse, which has been successfully used for isolation (Campbell and Domrow, 1974). It causes severe, highly invasive disease in several inbred strains of mice but manifests low toxicity for the adult mouse (Bell and Pickens, 1953). Growth characteristics are not appreciably different from those of related species. Identification, as with other species, is based on DNA sequence analysis or immunologic testing.

The disease in man is called Queensland tick typhus. *R. australis* was first isolated in 1944–1945 from the blood of two military patients on field exercises in belts of dense forest interspersed in grassy savannah in North Queensland, Australia (Andrew et al., 1946). Cases have also been recognized along the southeastern coast of Queensland and near Sydney. The scrub tick, *Ixodes holocyclus*, has been implicated in these infections. A strain was also isolated from *I. tasmani* collected from a wild rat (Campbell and Domrow, 1974).

Protein migration patterns are not appreciably different from those of related species (Pedersen and Walters, 1978). The genome size of *R. australis* is 1256–1276 kbp as determined by pulsed-field gel electrophoresis (Roux et al., 1992).

The mol% G + C of the DNA is: not known.

Type strain: NIAID Phillips 32.

GenBank accession number (16S rRNA): L36101, U12459.

The type strain was isolated from a patient by Andrew et al. (1946).

6. ***Rickettsia bellii*** Philip, Casper, Anacker, Cory, Hayes, Burgdorfer and Yunker 1983, 105^{VP}
belli'i. M.L. gen. n. *bellii* of Bell, named in honor of a rickettsiologist E. John Bell, who first isolated the organism.

The organisms are longer than most other rickettsiae with dimensions of $0.3\text{--}0.4 \times 2.0\text{--}3.0 \mu\text{m}$ during the log phase of growth and sometimes $10\text{--}15 \mu\text{m}$ under suboptimal conditions in tissue culture. Like other rickettsiae, *R. bellii* possess an outer slime layer; however, *R. bellii* can be differentiated from other rickettsiae because it has no discernible microcapsular layer. Most strains of *R. bellii* grow

poorly in yolk sacs although the type strain was originally isolated in embryonated eggs. Vero cell culture is a satisfactory system for primary isolation. Other cells such as *Xenopus laevis* (South African clawed toad) and tick cell lines have been used to isolate *R. bellii*. The cytopathic effect varies among isolates of *R. bellii*. Some isolates form characteristic and well defined but faint ring-shaped plaques, and others do not form plaques in Vero cells. Although *R. bellii* possesses antigens that cross-react with antibodies to *R. rickettsii*, *R. prowazekii*, and *R. typhi*, the protein composition of *R. bellii* differs from that of TG and SFG rickettsiae. Since 1974, 263 *R. bellii* isolates have been identified by immunofluorescence.

R. bellii has been detected in ticks from all geographic regions of the United States. In many locations, *R. bellii* is the predominant *Rickettsia* sp. in ticks. *R. bellii* comprises 39% (41/106) of isolates from *D. andersoni* in Montana, 82% (59/72) of isolates from North Carolina, and 83% (128/154) of isolates from Ohio. *R. bellii* has been identified in six species of ixodid ticks (*Dermacentor variabilis*, *D. andersoni*, *D. occidentalis*, *D. parumapertus*, *D. albipictus*, and *Hae-maphysalis leporispalustris*) and two species of argasid ticks (*Argas cooleyi* and *Ornithodoros concanensis*) (Philip et al., 1983).

A symbiont of a pea aphid (*Acyrtosiphon pisum*)—a plant-feeding insect—and an organism associated with a plant disease called papaya bunch top disease are phylogenetically placed in a clade with *R. bellii* (Chen et al., 1996; Davis et al., 1998).

Rickettsia bellii is nonpathogenic for mice and guinea pigs. Limited replication may occur in voles for some strains, but these strains cannot be maintained by serial passage in voles. There is no evidence that *R. bellii* causes human illness.

The mol% G + C of the DNA is: 30 (T_m). This value is similar to that of the TG rickettsiae but lower than that of the SFG rickettsiae.

Type strain: RML 369-C (Rocky Mountain Laboratories Collection).

GenBank accession number (16S rRNA): U11014.

RML 369-C (Rocky Mountain Laboratories Collection). This strain was isolated in embryonated chicken eggs from a triturated pool of unfed adult *Dermacentor variabilis* ticks collected from vegetation near Fayetteville, Arkansas, in June of 1966. After the fifth passage in yolk sacs, this organism regularly kills chicken embryos 4–5 days after inoculation (Philip et al., 1983).

7. ***Rickettsia canadensis*** McKiel, Bell and Lackman 1967, 509^{AL} (*Rickettsia canada* (sic) McKiel, Bell and Lackman 1967, 509.)

ca'na.den.sis. M.L. n. *canadensis* referring to Canada, the country where the organism was first isolated.

R. canadensis was previously known as *R. canada*. The name was corrected to *R. canadensis* because *canadensis* is the Latin adjective referring to Canada (Roux and Raoult, 2000).

R. canadensis can be grown in laboratory-reared ticks (Burgdorfer and Brinton, 1975), but grows sparingly in the louse (Weyer and Reiss-Gutfreund, 1973). Unlike some of the species of the spotted fever group, *R. canadensis* is not highly cytopathic and is cultivated in chicken embryos by the procedure used for the typhus group (Table BXII.α.31). Plaques on monolayers are small as are those of the typhus

group (Woodman et al., 1977). Virulence for the guinea pig and mouse is quite low. At least 10^6 viable cells are required to induce fever in the guinea pig, and 10^5 viable cells are required for seroconversion in mice (Ormsbee et al., 1978). The hemolytic activity as measured with sheep erythrocytes and the rate of CO_2 production from glutamate are approximately the same as those obtained with *R. prowazekii* and *R. typhi* (Woodman et al., 1977). *R. canadensis* reacts strongly with guinea pig and rabbit sera prepared against the typhus group rickettsiae but weakly with sera against spotted fever group rickettsiae; this weak reaction is due to the presence of epitopes in its LPS that are shared with TG rickettsiae prepared according to a particular immunization protocol (McKiel et al., 1967). Specific antigens can readily be demonstrated with mouse antisera (Philip et al., 1978). As with *R. prowazekii* and *R. typhi*, *R. canadensis* has antigens in common with *Proteus vulgaris* OX19, whereas SFG rickettsiae share antigens with the *Proteus vulgaris* OX2 antigen. Thus, *R. canadensis* was previously classified in the typhus group. However, *R. canadensis* resembles SFG rickettsiae in its other features, such as its ecological niche in the tick and its ability to grow in the nucleus of host cells. Moreover, *R. canadensis* has OmpA (Vishwanath, 1991), which is a common feature of SFG rickettsiae but not typhus rickettsiae. Thus, *R. canadensis* does not fit into either the TG or SFG of rickettsiae.

Two strains were isolated from pools of engorged rabbit ticks (*Haemaphysalis leporispalustris*) collected near Richmond, Ontario; one strain was isolated from an indicator rabbit, the other from a wild snowshoe hare (McKiel et al., 1967). Only one of these two strains is still available. Another strain was isolated in California from a *H. leporispalustris* tick feeding on a California jackrabbit (*Lepus californicus*) (Lane et al., 1981). There is serological evidence that human infection may have occurred (Bozeman et al., 1970).

Although the mol% G + C of the DNA of *R. canadensis* is identical to the values for *R. prowazekii* and *R. typhi*, the genome size is distinctly larger (1.49×10^9 Da). Thus, the extent of DNA–DNA hybridization between *R. canadensis* and the other two species of the typhus group varies somewhat owing to differences in the size of the genomes (38% and 46% hybridization with *R. prowazekii* and *R. typhi*, respectively). A degree of DNA–DNA hybridization occurs between *R. canadensis* and *R. rickettsii* that is similar to that between *R. canadensis* and *R. typhi* (Myers and Wiseman, Jr., 1981). When compared to *R. prowazekii* and *R. typhi* on the basis of migration patterns in polyacrylamide gel electrophoresis of solubilized proteins, *R. canadensis* displays a number of differences, although a basic similarity can still be recognized (Dasch et al., 1978).

The mol% G + C of the DNA is: 29 (T_m).

Type strain: 2678, ATCC VR-610.

GenBank accession number (16S rRNA): L36104.

8. *Rickettsia conorii* Brumpt 1932, 1199^{AL}

co.no'ri.i. M.L. gen. n. *conorii* of Conor; named after A. Conor who, in collaboration with A. Bruch, provided the first description of boutonneuse fever.

R. conorii resembles *R. rickettsii*, but it is antigenically distinct and less virulent for guinea pigs and humans; however, the Malish 7 strain is more virulent for C3H/HeN mice. Growth in cell cultures has the basic features of other rickettsiae of the spotted fever group, including early spread between host cells (Oaks and Osterman, 1979), and it is

cultivated by procedures identical to those used for *R. rickettsii*. This species can be differentiated from related species of the spotted fever group by appropriate DNA sequence analysis and serological tests (Bell and Stoenner, 1960; Bozeman et al., 1960). Differences among strains of *R. conorii* include different numbers, type, and order of tandem repeat units of OmpA. Genetic analyses reveal clustering of some isolates (e.g., those similar to Israeli isolates, those similar to Astrakhan isolates, and those similar to the Malish 7 South African isolate).

The human disease varies in severity and is fatal in 1–5% of hospitalized cases. It is called boutonneuse fever, Mediterranean spotted fever, Israeli tick typhus, Astrakhan spotted fever, Kenya tick typhus, Indian tick typhus, or other names that designate the locality of occurrence. It is normally transmitted by the bite of the tick, but may also be acquired through the skin or conjunctiva when the ticks are crushed. After a period of apparent low incidence, there was a marked rise in number of cases diagnosed during the 1970s.

R. conorii is the most geographically dispersed rickettsia of the spotted fever group. It has been recognized in most of the regions bordering on the Mediterranean Sea and Black Sea, Israel, Kenya and other parts of North, Central, and South Africa, and India. The brown dog tick, *Rhipicephalus sanguineus* is the prevailing vector. The involvement of rabbits in maintaining an infected tick population was suggested by association of the disease with large rabbit populations on endemic islands and by the drop in human disease following the myxomatosis epizootic that reduced the rabbit population in Europe (Hoogstraal, 1967, 1981).

The genome of *R. conorii* contains 1,268,755 bp (Ogata et al., 2001). No resident plasmid or bacteriophage has been identified in *R. conorii*. The electrophoretic profiles of the soluble proteins are similar but not identical to those of *R. rickettsii* and *R. sibirica* (Anacker et al., 1980).

The mol% G + C of the DNA is: 32.4 (determined from the genome sequence).

Type strain: Malish 7.

GenBank accession number (16S rRNA): AE008647 (genome segment), L36105 (Moroccan strain).

The type strain is a South African patient strain, isolated by J.H. Gear in 1946. This strain and the Moroccan strain (ATCC VR-141) are used most frequently in laboratory studies.

9. *Rickettsia felis* Bouyer, Stenos, Crocquet-Valdes, Moron, Popov, Zavala-Velazquez, Foil, Stothard, Azad and Walker 2001, 346^{VP}

fe'lis. L. gen. n. *felis* of the cat; named after the cat flea, *Ctenocephalides felis*, in which the organism was first observed by electron microscopy.

In 1990 during a study investigating potential vectors for *Neorickettsia risticii*, rickettsia-like organisms were observed in adult cat fleas obtained from EL Labs. Thus, the organism was originally designated as the ELB agent. The organisms are $0.25\text{--}0.45\ \mu\text{m} \times 1.5\ \mu\text{m}$ and are found in the midgut, tracheal matrix, muscles and reproductive tissues of the fleas. The organism contains a trilaminar cell wall that is characteristic of rickettsiae with a well-defined inner cell membrane and outer membrane. The dimensions of the microcapsular layer, outer and inner leaflets of the outer membrane, and the periplasmic space strongly resemble those in other *Rickettsia* species.

Phylogenetic analysis of the 17 kDa antigen gene, *ompA*, and *ompB* and other genes places *R. felis* as a member of the SFG.

R. felis has been cultivated in the XTC-2 cell line derived from *Xenopus laevis*. *R. felis* grows most rapidly at 28°C in XTC-2 cells, which die at a temperature of 32°C. The organism also grows to a lesser extent in Vero cells at 28°C or 32°C. L-929, and MRC-5 cell lines are unable to support the permanent growth of *R. felis*. Electron microscopy shows that *R. felis* is free in the cytoplasm but not in the nucleus of the cells.

The organism is maintained in the flea by transovarial transmission. Horizontal transmission or the acquisition of *R. felis* by fleas feeding on cats or artificially infected meals has not been demonstrated. *R. felis* has been associated with opossums and their fleas in Texas and California. Serological evidence and PCR detection indicate that *R. felis* infection occurs in humans in the United States, Mexico, Brazil, and Germany. The organism has also been detected in cat fleas from Spain and Ethiopia; thus, *R. felis* is likely distributed worldwide.

The mol% G + C of the DNA is: not determined.

Type strain: CNCM I-2363, Marseille-URRWFxCal2 (Reference Center for Rickettsioses, Marseille, France).

10. **Rickettsia helvetica** Beati, Peter, Burgdorfer, Aeschlimann and Raoult 1993, 524^{VP}
hel.vet.ti.ca. N.L. adj. *helvetica* pertaining to Helvetia, the Latin name of Switzerland where the organism was originally isolated.

R. helvetica, formerly called the Swiss agent, was first isolated from *Ixodes ricinus* ticks from Switzerland (Burgdorfer et al., 1979). Recently, *R. helvetica* has been isolated from *Ixodes ricinus* ticks from France (Parola et al., 1998). Rickettsial isolates from *Ixodes ovatus*, *I. persulcatus*, and *I. monspinosus* from Japan are identical or closely related to *R. helvetica* (Fournier et al., 2002). In serological tests, *R. helvetica* antigens are weakly cross-reactive with antisera to other SFG rickettsiae.

R. helvetica grows well in yolk sacs of embryonated eggs, chicken embryo fibroblasts, L929, and Vero cells. Human embryonic lung fibroblasts do not support the growth of *R. helvetica* in primary isolation. *R. helvetica* does not cause a cytopathic effect in Vero cells, and it can be continuously subcultured by trypsinization of the monolayers. Unlike other SFG rickettsiae, *R. helvetica* does not stimulate actin tail formation when it grows inside the host cell. *R. helvetica* exhibits moderate pathogenicity in meadow voles; however, rabbits, guinea pigs, and mice are not susceptible (Fournier et al., 2000). A serological survey suggests that a portion of tick-exposed humans have antibodies reactive with *R. helvetica* in France (Nilsson et al., 1999). However, the pathogen has not been isolated from ill humans.

The mol% G + C of the DNA is: not determined.

Type strain: C3 (Reference Center for Rickettsioses, Marseille, France), ATCC VR-1375.

GenBank accession number (16S rRNA): L36214.

11. **Rickettsia honei** Stenos, Roux, Walker and Raoult 1998, 1403^{VP}
ho.ne.i. L. gen. n. *honei* of Hone, named after Frank Sandland Hone, an early pioneer in Australian rickettsiology.

An SFG rickettsiosis-like ailment was identified in 1991 on Flinders Island of Australia and was named Flinders

Island spotted fever (FISF). The causative agent of FISF was isolated from buffy coat preparations from the blood of two patients (Baird et al., 1992) and named *R. honei* (Stenos et al., 1998).

R. honei and the Thai tick typhus rickettsia (TT-118) are identical in DNA sequences of the gene encoding the 17 kDa protein, *gltA*, and *ompA*, and they have only 1 nucleotide difference in the 16S rRNA gene. Thus, *R. honei* and TT-118 are considered a single species (Stenos et al., 1998).

TT-118 was isolated from a pool of immature *Rhipicephalus* and *Ixodes* of unknown species collected in Thailand (Robertson and Wisseman, 1973). A *Rickettsia* identical to TT-118 has recently been detected by PCR from an *Ixodes granulatus* tick from Thailand (Kollars et al., 2001). Migratory birds have been postulated to spread *R. honei* widely. A rickettsia genetically identical to *R. honei* has been detected by PCR in *Aponoma hydrosauri* ticks from Flinders Island and Tasmania. This tick is a reptile-associated tick, but it also bites humans (Whitworth et al., 2003).

The mol% G + C of the DNA is: not determined.

Type strain: RB, ATCC VR-1472.

GenBank accession number (16S rRNA): U17645, AF060705.

The type strain of *R. honei* was isolated from buffy coat of a patient's blood by inoculation of buffalo green monkey kidney cells.

12. **Rickettsia japonica** Uchida, Uchiyama, Kumano and Walker 1992, 303^{VP}
ja.po.ni.ca. N.L. adj. *japonica* pertaining to Japan, the country from which the first isolates were identified.

The dimensions of the organisms are 0.4–0.5 × 0.8–1.5 µm. Unlike some other SFG rickettsiae, *R. japonica* can persistently infect Vero cells. *R. japonica* does not cause a cytopathic effect in Vero cells but does cause cytopathic effects in L929, BHK21/13, and primary chicken embryo fibroblast cells. The rickettsiae grow primarily in cytoplasm and rarely in nuclei. The plaque morphology produced by *R. japonica* differs from that produced by most other SFG rickettsiae except *R. rickettsii*. *R. japonica* forms target like plaques (a dye-stained inner focus of viable cells surrounded by a clear zone of necrotic cells) after 9–11 days of incubation in Vero cells. Other SFG rickettsiae form clear plaques. A variant that forms clear plaques was isolated from a guinea pig inoculated with *R. japonica*. About 0.01 to 0.001% of cells carrying strain YH produced clear plaques on Vero cells (Uchida et al., 1992).

The organisms are pathogenic for guinea pigs. Guinea pigs develop fever and scrotal swelling. C3H/He strain of inbred mice and conventional strain ddY mice are resistant to *R. japonica* infection. Chicken embryos die 5–7 days after yolk sac inoculation. Like other SFG rickettsiae, *R. japonica* does not exhibit hemolytic activity.

R. japonica is presumably transmitted by ticks. It has been cultivated from *Dermacentor taiwanensis* and *Haemaphysalis flava* (Fournier et al., 2002).

The disease caused by *R. japonica* infection is Japanese spotted fever. The disease was first found in Japan in 1984. The disease is mainly distributed along the coast of southwestern and central Japan where the climate is warm, and most cases occur between April and October. The clinical features of the disease include fever (100%), headache (80%), shaking chills (87%), skin eruption (100%), and tick bite eschars (90%) (Mahara, 1997). Serologic evidence

indicates that disease might occur in eastern Asia and adjacent islands (Feng et al., 1991; Camer et al., 2000). However, the antibodies against *R. japonica* might have been stimulated by other SFG rickettsiae in these areas.

"*R. heilongjiangii*", a rickettsial isolate from both ticks and humans in northeastern China, is closely related to *R. japonica*. The 16S rRNA gene sequence of "*R. heilongjiangii*" has 99.6% similarity to that of *R. japonica* (Zhang et al., 2000b). Further studies need to be performed to determine whether "*R. heilongjiangii*" should be designated a strain of *R. japonica*.

The mol% G + C of the DNA is: 31.2 (T_m).

Type strain: YH; ATCC VR-1363.

GenBank accession number (16S rRNA): L36213 (strain YM).

The YH strain was isolated from the blood of a patient in Japan by Uchida et al. (1985).

13. **Rickettsia massiliae** Beati and Raoult 1993, 839^{VP}
mas.si'li.ae. L. gen. n. *massiliae* from Massilia, the Latin name of Marseille, where the organism was first isolated.

The dimensions of the organisms are $0.3\text{--}0.4 \times 0.6\text{--}1$ μm . Growth occurs in the cytoplasm of eucaryotic cells. *R. massiliae* can be cultivated in L929, Vero, or human embryonic lung fibroblast cells. *R. massiliae* has been isolated from *Rhipicephalus turanicus* and *R. sanguineus* ticks in France. *R. massiliae* has also been detected in ticks from Greece, Portugal, Spain, and the Central African Republic where *Rhipicephalus lunulatus*, *R. mushamae*, and *R. sulcatus* are hosts (Beati and Raoult, 1993; Babalis et al., 1994; Beati et al., 1996). Pathogenicity of *R. massiliae* for humans and animals has not been demonstrated.

The mol% G + C of the DNA is: not determined.

Type strain: Mtu1, ATCC VR-1376.

GenBank accession number (16S rRNA): L36214.

Mtu1 was isolated from the hemolymph of a *Rhipicephalus turanicus* tick collected in Marseille, France (Reference Center for Rickettsioses, Marseille, France).

14. **Rickettsia montanensis** corrig. (ex Lackman, Bell, Stoenner and Pickens 1965) *Rickettsia montana* (sic) Weiss and Moulder 1984b, 356^{VP} (Effective publication: Weiss and Moulder 1984a, 697.)
mon.tan'en.sis. M.L. gen. n. *montanensis* from Montana, the state where the organism was first isolated.

Resembles other members of the spotted fever group in its growth characteristics in the chicken embryo and antigenic composition, but it is avirulent for the guinea pig and mouse. Although antibodies reactive with this species have been detected in dogs, *R. montanensis* is not associated with signs or symptoms of spotted fever in humans or experimentally inoculated dogs. It is distinguished from other rickettsiae by DNA sequence analysis and immunologic testing (Philip et al., 1978). *R. montanensis* was first isolated in eastern Montana, repeatedly from *D. variabilis*, occasionally from *D. andersoni*. It has been also isolated with notable frequency from rodents (genera *Microtus* and *Peromyscus*) and from *D. variabilis* in various parts of the U.S. (Lackman et al., 1965; Philip et al., 1978; Linnemann et al., 1980).

The mol% G + C of the DNA is: not determined.

Type strain: ATCC VR-611.

GenBank accession number (16S rRNA): L36099.

ATCC VR-611 is the tick strain of Bell et al. (1963).

15. **Rickettsia parkeri** Lackman, Bell, Stoenner and Pickens 1965, 137^{AL}

par'ke.ri. M.L. gen. n. *parkeri* of Parker; named after Ralph R. Parker, a founder of the Rocky Mountain Laboratory.

This species is cultivated in eggs by the same procedure used for the other species of the spotted fever group. *R. parkeri* is identified by DNA sequence analysis or immunologic evaluation. *R. parkeri* produces a nonfatal disease in the guinea pig that is characterized by fever and reddening of the scrotum; the most abundant growth takes place in the testicular tissue (Parker et al., 1939). Human infection has not been reported. The species has been isolated from *Amblyomma maculatum* ticks collected from domestic animals in Texas, Georgia and Mississippi (Lackman et al., 1949).

The mol% G + C of the DNA is: not determined.

Type strain: NIAID maculatum 20.

GenBank accession number (16S rRNA): U12461.

The type strain was isolated in 1948 from *A. maculatum* ticks collected from sheep in Mississippi (Bell and Pickens, 1953).

16. **Rickettsia peacockii** Niebylski, Schrupf, Burgdorfer, Fischer, Gage and Schwann 1997b, 451^{VP}

pea.cock'i.i. M.L. gen. n. *peacockii* of Peacock, named after M.G. Peacock, a well-respected rickettsiologist.

R. peacockii is a endosymbiont of wood ticks (*Dermacentor andersoni*) collected from the Bitterroot Valley in the western Montana. *R. peacockii* is transstadially and transovarially transmitted in ticks. Massive infections of *R. peacockii* are observed only in *D. andersoni* ovarian tissues. The organisms are not found in tick hemocytes, salivary glands, midgut, Malpighian tubules, or hypodermal tissues. Thus, *R. peacockii* is likely unable to be transmitted to mammals.

The 16S rRNA gene sequence is most similar to *R. rickettsii* R strain and *R. slovaca* (99.7% for both species). The sequence of the *ompA* gene is most similar to *R. slovaca* (93.0%). The *ompA* gene of *R. peacockii* has a single nucleotide deletion at position 334. The deletion results in a prematurely truncated OmpA of *R. peacockii* with 110 amino acids only. Thus, presumably *R. peacockii* does not have a functional OmpA (Niebylski et al., 1997b).

R. peacockii is postulated to interfere with the stable maintenance of virulent rickettsiae in nature (Burgdorfer et al., 1981) because the distribution of *R. peacockii* coincides with a low incidence of Rocky Mountain Spotted Fever (RMSF) on the east side of the Bitterroot Valley, where early cases of RMSF were recognized on the west side of the valley. *R. peacockii* infects approximately 70% of wood ticks from the east side but is uncommon in the ticks from the west side of the valley. A plausible explanation for the *R. peacockii* interference is that infections of *R. peacockii* in tick ovarian tissues might inhibit transovarial transmission of virulent *R. rickettsii* (Niebylski et al., 1997b).

The mol% G + C of the DNA is: not determined.

Type strain: Skalkaho.

GenBank accession number (16S rRNA): U55820.

This strain was characterized directly from an infected *D. andersoni* tick (SK-594) that was used to generate the 16S rRNA gene sequence and *ompA* gene sequence. The organism has been recently co-cultured from the *D. andersoni* cells (Simser et al., 2001).

17. **Rickettsia rhipicephali** (ex Burgdorfer, Brinton, Krynski and Philip 1978) Weiss and Moulder 1988, 221^{VP} (Effective publication: Weiss and Moulder 1984a, 698.)
rhi.pi.ce'pha.li. M.L. gen. n. *rhipicephali* of *rhipicephalus*; named after its natural tick host *Rhipicephalus sanguineus*.

Although this species is isolated with difficulty in the chicken embryo and the guinea pig, it is readily recovered in male meadow voles (*Microtus pennsylvanicus*), in which it produces massive infection in the tissues of the tunica vaginalis. This species can also be cultivated on monolayers of chick embryo fibroblasts, Vero, and mouse L cells. Although growth is profuse, damage to the cells is limited, and plaques are small and turbid, reflecting a mixture of viable and dead host cells. There is no evidence that *R. rhipicephali* is pathogenic for humans. A serosurvey of acute and convalescent serum samples from 80 dogs in which Rocky Mountain spotted fever had been considered as a differential diagnosis identified one dog with a fourfold increase in antibody titer to *R. rhipicephali* (Breitschwerdt et al., 1995). *R. rhipicephali* has been detected in about 19% of the brown dog ticks (*R. sanguineus*) removed from dogs in central and northern Mississippi and from ticks collected in Texas and North Carolina (Burgdorfer and Brinton, 1975; Burgdorfer et al., 1978). It has also been isolated with high frequency from *Dermacentor andersoni* ticks collected in western Montana (Philip and Casper, 1981). The genome size of *R. rhipicephali* is 1252–1258 kb as determined by pulsed-field gel electrophoresis (Roux et al., 1992).

The mol% G + C of the DNA is: 32.2 (T_m) (Anacker et al., 1980).

Type strain: Burgdorfer 3-7-female 6.

GenBank accession number (16S rRNA): L36216.

This strain was isolated in 1973 in Mississippi by Burgdorfer et al. (Burgdorfer et al., 1975).

18. **Rickettsia rickettsii** (Wolbach 1919) Brumpt 1922, 757^{AL} (*Dermacentroxenus rickettsii* Wolbach 1919, 87.)
rickett'si.i. M.L. gen. *rickettsii* of Ricketts; named after Howard Taylor Ricketts for his classic studies of the etiology of Rocky Mountain spotted fever.

R. rickettsii is the most widely studied species of the spotted fever group. The cells are slightly smaller and more uniform in size than those of *R. prowazekii*.

Because of the high virulence of *R. rickettsii* for chicken embryos, the embryos die before extensive growth has taken place, and the procedure for optimal harvest from yolk sacs differs considerably from the one employed for the typhus group (Table BXII.α.31). It is performed as follows (Stoennner et al., 1962). Embryos, 4–5 d old, are inoculated with a sufficient number of viable cells to kill most embryos within 4–5 d. The eggs are incubated at 33.5°C (lowest temperature compatible with survival of most embryos) and maintained in an incubator at 32°C for 2 d after death of the embryos. Even under the best conditions, yields of *R. rickettsii* cells from yolk sac are smaller than those of *R. prowazekii* or *R. typhi*, but they are somewhat higher than yields obtainable from cell cultures.

Multiplication occurs primarily in the cytoplasm, but intranuclear growth is sufficiently prominent to have stimulated early investigators to use it as a criterion for the classification of the spotted fever group. Because of cell-to-cell spread via actin-based mobility and greater cytopathogenicity, plaques on monolayers occur earlier and are larger

than those formed by the typhus group; however, sheep or rabbit erythrocytes are not hemolyzed (Table BXII.α.31).

Strains of this species vary considerably in virulence for the guinea pig. Virulence for humans and guinea pigs appears to vary independently.

The guinea pig is highly susceptible to infection. The more virulent strains induce fever and scrotal necrosis, and the infection is often fatal. In humans, *R. rickettsii* is the causative agent of Rocky Mountain spotted fever, the most severe disease of the spotted fever group. The disease is characterized by high fever and widespread damage to the small blood vessels; this damage results in a skin rash, increased vascular permeability associated with non-cardiogenic pulmonary edema and vascular lesions in various organs including encephalitis. Before the era of the tetracycline and chloramphenicol antibiotics, the case fatality rates were as high as 90% in the Bitterroot Valley of Montana and as low as 5% in the Snake River Valley of Idaho and 25% in Long Island. Because the disease responds well to tetracyclines, present mortality rates are relatively low, provided the disease is recognized and treated promptly. Dogs are susceptible to natural infection and develop clinical signs (Lissman and Benach, 1980). The mouse is quite resistant to infection, and doses in excess of 10⁶ viable cells are required to produce a significant antibody response.

The metabolism of *R. rickettsii* is similar to that of other rickettsiae. Biochemical investigations have focused on loss and restoration of viability, and metabolic activity is best maintained under microaerophilic conditions or in the presence of reduced glutathione or protein. Reversible changes in rickettsial activity occur in the tick: prolonged refrigeration reduces the virulence of the rickettsiae for the guinea pig, but virulence is restored when the ticks have a blood meal or are incubated at 37°C for 24–48 h. Spencer and Parker (1923) postulated that virulence of *R. rickettsii* in the tick vector is linked directly to the physiological state of the tick and defined this phenomenon as “reactivation.” The reactivation may result from growth of rickettsiae or differential expression of rickettsial virulence factors at the elevated temperature or after stimulation by components of the blood meal. *R. rickettsii* numbers increase 100-fold in the hemolymph of partially engorged ticks compared to unfed infected ticks (Wike and Burgdorfer, 1972). Differential expression of rickettsial proteins and ultrastructural changes has been confirmed by immunoblot and electron microscopy. Electron microscopy reveals that reactivated *R. rickettsii* in ticks incubated at 37°C or in ticks fed on animals has a discrete microcapsular layer and a discrete electron-lucent slime layer outside the microcapsular layer. In starved ticks, the microcapsular layer and the slime layer of rickettsiae are inconspicuous or ragged (Hayes and Burgdorfer, 1982). Immunoblot analysis indicates that rickettsial proteins of 42, 43, 48, 75 and 100 kDa are induced in a tick cell line when the incubation temperature is raised from 28°C to 34°C (Policastro et al., 1997).

In a series of brilliant experiments conducted in 1906 and 1907, Ricketts (1911) clearly established the basic features of the ecology of the agent of Rocky Mountain spotted fever in the tick. *R. rickettsii* is confined to the Western Hemisphere (Table BXII.α.31), but at present, it is encountered much more frequently in the southeastern and central United States, particularly in North Carolina and Oklahoma, than in the Rocky Mountains. In the western

United States, the most common human vector is the wood tick, *Dermacentor andersoni*. In the eastern two thirds of the United States and parts of the Far West, the American dog tick, *Dermacentor variabilis*, is the chief vector. The brown dog tick, *Rhipicephalus sanguineus*, and the cayenne tick *Amblyomma cajennense* have been implicated in transmission of human infections in Mexico and South America.

Although there is considerable serological evidence of widespread exposure to SFG rickettsiae among wild vertebrates and domestic dogs, demonstration of infection by recovery of the microorganisms has been difficult, presumably because sufficient numbers of rickettsiae are present in the vertebrate host only during brief periods. Natural infection among vertebrates was first demonstrated in the 1930s in Brazil in domestic and wild dogs (*Canis brasiliensis*), the opossum (*Didelphis marsupialis*), the wild rabbit (*Sylvilagus minensis*), and the Brazilian capybara (*Cavia aperea*) (Moreira and de Magalhaes, 1937). Recovery of *R. rickettsii* has been reported in the United States (Gould and Miesse, 1954) from the meadow vole (*Microtus pennsylvanicus*), opossum, cotton rat (*Sigmodon hispidus*), cottontail rabbit (*Sylvilagus floridanus*), whitefooted mouse (*Peromyscus* sp.) and pine vole (*Pitymys pinetorum*) (Bozeman et al., 1967). In the western United States, *R. rickettsii* has been isolated from the chipmunk (*Eutamias amoenus*), snowshoe hare (*Lepus americanus*) and the golden-mantled ground squirrel (*Citellus lateralis tescorum*) (Burgdorfer et al., 1962). Ricketsemic cotton rats have been demonstrated experimentally as a source of *R. rickettsii* for *D. variabilis* ticks, and cotton rats are susceptible to the infection when transmitted by tick bite.

Transmission to man occurs through the bite of an infected tick that remains attached to the skin for several hours. Although man is only an incidental host, human infection reflects a changing ecology. From 1910 to 1930 most of the cases, about 100–600 per year, were diagnosed within the area of distribution of *D. andersoni* in the Rocky Mountain region. Since 1930, there has been a decrease in incidence in the Rocky Mountain region along with increased recognition of the disease in the southeastern parts of the United States associated with transmission by *D. variabilis*. From 1948 to 1959, the number of cases decreased from an annual rate of 500 to an annual rate of 200. After 1959, the disease increased to an annual rate greater than 1000 cases by 1977. A decrease in reported cases occurred during the 1980s and early 1990s, but there were hints of an increase in the late 1990s. A strain of *R. rickettsii* has been isolated from patients in Costa Rica (Fuentes, 1979).

The genome is 1,257,710 bp in size and contains 1486 open reading frames and 33 tRNA genes (Eremeeva et al., 2002). The proteins of various strains of *R. rickettsii* have shown no differences except for minor epitope differences of the HLP strain, an isolate that has been identified only in ticks (Anacker et al., 1980).

The mol% G + C of the DNA is: 32.4 (determined from the genome sequence).

Type strain: ATCC VR-149.

GenBank accession number (16S rRNA): L36217.

Strain Sheila Smith was isolated from a patient in Montana (Bell and Pickens, 1953). Reference strain: strain R, was isolated from *Dermacentor andersoni*.

This species closely resembles *R. rickettsii*, although most strains are less virulent for animals and humans. Cinematographic observations by Kokorin et al. (1978) have revealed intense mobility of the rickettsiae within their host cells, and some movement from cell to cell, but only limited cytotoxicity occurs. The species is cultivated by procedures identical to those used for *R. rickettsii*. Virulence for the guinea pig is quite variable, and in some cases, only fever of short duration is produced. An occasional strain kills mice and hamsters (Bazlikova and Brezina, 1978), and inbred strains of mice vary in susceptibility to this organism (Kekcheeva et al., 1978). The disease in humans resembles moderately severe or mild Rocky Mountain spotted fever. It is called Siberian or North Asian tick typhus.

This species is differentiated from the other strains of the spotted fever group by DNA sequence analysis of *ompA*, *ompB*, the 17 kDa lipoprotein gene, the citrate synthase gene, or the 120 kDa cytoplasmic antigen gene. Differentiation can also be achieved by immunologic analysis using IFA titration with sera prepared according to a particular protocol in mice (Philip et al., 1978). Toxicity neutralization tests in mice demonstrate the close relationship of *R. sibirica* with *R. rickettsii* (Lackman et al., 1965).

The habitat of *R. sibirica* consists of foci extending from the Pacific Coast of Russia and China in the east to Armenia in the west and from Siberia in the north to northern China in the south. The foci are usually associated with steppe landscapes with low rainfall close to foothills and mountain ranges, and they may extend to the dry slopes of the mountains. *R. sibirica* has been detected in ticks of the following genera: *Demacentor* (*D. nuttalli*, *D. marginatus*, and *D. silvarum*), *Haemaphysalis* (*Ha. punctata*, *Ha. concinna*, and *Ha. japonica*), *Hyalomma* (*Hy. dromedarii*, *Hy. asiaticum*, *Hy. detritum*, *Hy. anatolicum*, *Hy. plumbeum*, *Hy. marginatus*), *Ixodes* (*I. ricinus*, *I. persulcatus*, *I. apronophorus*, and *I. plumbeus*), and *Rhipicephalus* (*R. sanguineus*, *R. turanicus*, and *R. schulzei*). These ticks feed on birds, numerous small wild rodents, and domestic animals. Antibodies to *R. sibirica* have been detected from at least 18 kinds of mammals, including Siberian squirrels or susliks (genus *Citellus*), chipmunks (*Eutamias*), hamsters (*Cricetus*), lemmings (*Lagurus*), hares (*Lepus*), domestic and field mice, and voles (Hoogstraal et al., 1967). *R. sibirica* has been isolated from wild rodents and birds but not from domestic animals. Rodents are the main seasonal reservoir of *R. sibirica* in its natural focus, and birds may serve as the vehicle to establish new foci over long distances. Humans are infected through the bite of an infected tick.

The genome size is 1,255,665 bp in size and contains 1316 open reading frames and 33 tRNA genes (Eremeeva et al., 2002). The electrophoretic migration patterns in polyacrylamide gel of the solubilized proteins are similar but not identical to those of *R. rickettsii* (Pedersen and Walters, 1978).

The mol% G + C of the DNA is: 32.4 (determined from the genome sequence).

Type strain: 246, ATCC VR-151.

GenBank accession number (16S rRNA): L36218.

Strain 246 was isolated from *Dermacentor nuttalli* in the U.S.S.R. about 1949 (Bell and Stoenner, 1960).

19. *Rickettsia sibirica* Zdrodovskii 1949, 20^{AL}

si.bi'ri.ca. M.L. fem. adj. *sibirica* pertaining to Siberia.

20. *Rickettsia slovaca* Sekeyova, Roux, Xu, Reháček and Raoult 1998, 1458^{VP}

slo.va.ca'. L. gen. n. *slovaca* from Slovakia, the country where the organism was first isolated.

The dimensions of the organisms are $0.37\text{--}0.45 \times 0.8\text{--}1.2 \mu\text{m}$. *R. slovaca* can be propagated in yolk sacs of chicken embryos, HEL, Vero, L929, and tick cells. No cytopathologic effect is observed in any of the infected cell lines. Electron microscopy reveals that the organisms are free in the host cell cytoplasm and are surrounded by an electron-lucent zone. The organism has very low pathogenicity for mice and guinea pigs. The only observed change in inoculated mice is a slightly enlarged spleen. Guinea pigs develop brief and mild fever after inoculation of the organisms. *R. slovaca* causes asymptomatic infection in *Lepus europaeus* and hamsters. *R. slovaca* has been associated with human infections that are characterized as usually afebrile with an eschar and regional lymphadenopathy after the bite of a *D. marginatus* or *D. reticulatum* tick. *R. slovaca* was isolated from a pool of male and female *Dermacentor marginatus* ticks collected in Slovakia. Rickettsial strains closely related to *R. slovaca* have been isolated from Armenia, Crimea, France, Portugal, Switzerland, Ukraine, and Yugoslavia, in the geographic distribution of *D. marginatus* and *D. reticulatum* (Sekeyova et al., 1998).

The mol% G + C of the DNA is: not determined.

Type strain: B (Reference Center for Rickettsioses, Marseille, France).

21. **Rickettsia typhi** (Wolbach and Todd 1920) Philip 1943, 304^{AL} (*Dermacentroxenus typhi* (Wolbach and Todd 1920) *Rickettsia mooseri* Monteiro 1931, 97.)
ty'phi. Gr. n. *typhus* cloud, hence stupor associated with rickettsial encephalitis; M.L. n. *typhus* fever, typhus; M.L. gen. n. *typhi* of typhus.

R. typhi is the valid name for the species, appearing on the Approved Lists of Bacterial Names (Skerman et al., 1980). However, the species has also been called "*R. mooseri*" in honor of Herman Mooser, who clearly differentiated the species from *R. prowazekii* based on its virulence for the guinea pig (Mooser, 1928).

R. typhi is more virulent than *R. prowazekii* for guinea pigs and mice (Table BXII.α.31). Unlike *R. prowazekii*, it can be passed indefinitely in the rat and may persist for months in the rat brain. Rats (*Rattus norvegicus* and *R. rattus*, especially those located in urban areas) and other rodents are the primary reservoirs. *R. typhi* has a worldwide distribution and has been reported from nearly all countries where investigators have competently searched for it. The rat louse, *Polyplax spinulosus*, and the rat flea, *Xenopsylla cheopis*, are the chief transmitters of the organism from rat to rat. The human flea, *Pulex irritans*, and the human body louse are highly susceptible to infection and may play roles in transmission in populations with high ectoparasitic infestation. The rat-rat flea cycle has been interrupted in the United States by control measures, including killing of fleas with DDT. Currently the principal *R. typhi* maintenance cycle in the United States involves the cat flea (*Ctenocephalides*

felis) and opossums (*Didelphis virginianus*), and the cat flea is the apparent vector for human infections.

The human disease is a mild form of typhus and is called murine or endemic typhus, but it is also known by names that reflect the geographic location. "Tabardillo" in Mexico might be murine typhus transmitted by lice. Murine typhus is reported with variable frequency, undoubtedly largely because of DDT interruption of the transmission by fleas; for instance, in the United States, a peak of 5400 cases occurred in 1944, but the incidence was less than 100 cases in 1958. Many cases are not properly documented (Traub et al., 1978), due in part to effective treatment of the disease with antibiotics.

Some of the antigens of *R. typhi* demonstrable in serological tests are very similar, if not identical, to those of *R. prowazekii*, while others are species-specific. The antigens containing common epitopes are numerous and include LPS, OmpB, the 17kDa proteins, GroES and GroEL. OmpB-containing species-specific epitopes as well as 10–15% of the total cellular protein are released as a soluble fraction when the cells are suspended in a hypotonic solution that lacks Mg^{2+} and is incubated at 45°C for 20 min (Dasch, 1981). The two species can also be distinguished immunologically by species-specific monoclonal antibodies; cross-absorption of defined antisera and subsequent IFA titration or immunoblotting; cross-challenge of guinea pigs vaccinated with formalin-inactivated antigens; or mouse toxicity neutralization tests.

There have been no extensive attempts to isolate mutant strains of *R. typhi* in the laboratory. Differences between *R. typhi* and *R. prowazekii* in cell morphology and in the mechanism of interaction with eucaryotic cells undoubtedly exist, such as the relatively ineffective actin-based mobility of *R. typhi* as compared to absence of actin-based mobility of *R. prowazekii*; however, objective criteria of differentiation based on such characteristics have not been developed. Differences in metabolic activities have not been described, and the two species have been used interchangeably for metabolic investigations.

The mol% G + C of the DNA of *R. typhi* is similar to that of *R. prowazekii*. The genome size of *R. typhi* is $1,133 \pm 44 \text{ kb}$ (Eremeeva et al., 1993). Although no differences in the degree of DNA–DNA hybridization have been found between strains of *R. typhi*, the degree of hybridization between *R. typhi* and *R. prowazekii* is 70–79%, (Myers and Wiseman, 1980). Similarly, no differences in electrophoretic protein migration patterns have been noted between strains of *R. typhi*, but consistent differences in the migration patterns of malate dehydrogenase and of several unidentified proteins have been found between *R. typhi* and *R. prowazekii* (Dasch et al., 1978).

The mol% G + C of the DNA is: 29 (T_m).

Type strain: Wilmington, ATCC VR-144.

GenBank accession number (16S rRNA): M21789.

Strain ATCC VR-144 is that of Maxcy (1929).

Other Organisms

1. "*Rickettsia amblyommii*"

A rickettsial agent that was first described by Burgdorfer et al. (1974) was later characterized as a new SFG rickettsia and referred to as WB-8-2 (Burgdorfer et al., 1981). WB-

82-2 was isolated from an *Amblyomma americanum* tick collected from vegetation in Tennessee (Burgdorfer et al., 1974). The organism invades all tick tissues but, in general, produces a mild to moderate infection except in the ovary

where it may be abundant and from where it is passed via eggs to as many as 100% of progeny. The organism grows well in avian cell cultures but very poorly in embryonated hen's eggs, which die 5–7 days after inoculation. WB-8-2 is nonpathogenic for guinea pigs. In male meadow voles (*Microtus pennsylvanicus*), it produces microscopically detectable mild and transient infections in the tunica vaginalis only after inoculation of heavily infected tissue culture suspensions. Epidemiological evidence suggests that WB-8-2 is nonpathogenic for humans (Burgdorfer et al., 1981). Weller et al. (1998) isolated another strain of this rickettsia (MOAa) from an adult female *A. americanum* tick collected in Bolling County, Missouri. The MOAa organism is highly cytopathogenic for RAE25 cells, a tick cell line. Phylogenetic analysis indicates that strains MOAa and WB-8-2 are closely related. They represent different strains of "*Rickettsia amblyommii*". Strains MOAa and WB-8-2 are most closely related to *R. montanensis* (Weller et al., 1998).

2. "*Rickettsia cooleyi*"

A rickettsial agent exists in *Ixodes scapularis* ticks collected from Anderson County in eastern Texas. The organism has not yet been cultivated. It was identified by amplification of rickettsial genes by the polymerase chain reaction. DNA sequencing showed the highest nucleotide sequence similarity with *R. australis* for the 17-kDa protein gene, with *R. helvetica* for *gltA*, and with *R. montanensis* for *ompA*. The organism was provisionally designated as the Cooleyi agent after Dr. Robert A. Cooley, who served the Montana State Board of Entomology from 1899 to 1944 (Billings et al., 1998). Weller et al. (1998) have also sequenced *ompA* of a symbiont in ovarian tissues of *I. scapularis*. The *ompA* se-

quences of the *I. scapularis* symbiont and "*Rickettsia cooleyi*" are identical.

Species Incertae Sedis

1. "*Rickettsia monacensis*" Simser, Palmer, Fingerle, Wilske, Kurti and Munderloh 2002.

mo.na.cen'sis. M.L. n. *Monacum* Munich, a German city; M.L. adj. *monacensis* from/of Munich.

The name has been proposed for a rickettsial agent that exists in *Ixodes ricinus* collected in the English Garden in Munich, Germany (Simser et al., 2002). The cell size is $1.0\text{--}1.5 \times 0.3\text{--}0.4 \mu\text{m}$. The organisms resemble SFG rickettsiae in their ultrastructure, and they reside free in the cytoplasm and occasionally within the nuclei of host cells. The slime layer is thinner ($<30 \text{ nm}$) than that of *R. rickettsii* (30–60 nm). This rickettsia was originally isolated by using a tick cell line (ISE6) and subsequently cultivated in L-929 cells. The organisms are cytopathic for ISE6 cells and cause cell lysis after the third passage. The rickettsia induces polymerization of host actin in both tick and mammalian cells. The immunodominant proteins are OmpA and OmpB. Phylogenetically the organism is distantly related to other SFG rickettsiae. Its pathogenicity for animals and humans is unknown.

The mol% G + C of the DNA is: not determined.

Deposited strain: Type strain: IrR/Munich.

Strain IrR/Munich was isolated from a female *I. ricinus* tick (deposited by U. Munderloh at the Rickettsial and Ehrlichial Diseases Research Laboratory, University of Texas Medical Branch, Galveston, Texas, USA).

Genus II. *Orientia* Tamura, Ohashi, Urakami and Miyamura 1995, 590^{VP}

XUE-JIE YU AND DAVID H. WALKER

O'ri.en'ti.a. M.L. fem. n. *Orientia* pertaining to the Orient, the area where the organisms are widely distributed.

Short rods $0.5\text{--}0.8 \times 1.2\text{--}3.0 \mu\text{m}$. **Obligately intracellular. Grow free in the cytosol of the host cell;** rarely invade the nucleus. Bacterial cells are released surrounded by host cell membrane. **Cell walls lack lipopolysaccharide and peptidoglycan.** The outer leaflet of the cell wall is considerably thicker than the inner leaflet, unlike the wall of rickettsiae. The genome of *Orientia* is $1.1\text{--}1.5 \times 10^9 \text{ Da}$, larger than that of *Rickettsia*. Maintained by transovarian transmission in its various **trombiculid mite hosts**. **Etiological agents of scrub typhus in humans.** Transmitted to humans by mite larvae. Found in Asia and Australia.

The mol% G + C of the DNA is: 28.1–30.5 (HPLC).

Type species: Orientia tsutsugamushi (Hayashi 1920) Tamura, Ohashi, Urakami and Miyamura 1995, 590 (*Rickettsia tsutsugamushi* (Hayashi 1920) Ogata 1931, 252; "*Theileria tsutsugamushi*" Hayashi 1920, 63.)

FURTHER DESCRIPTIVE INFORMATION

16S rRNA gene analysis indicates that the organisms are separate from the *Rickettsia* species, with a similarity value of 90.2–90.6%. This genus contains a single species, *O. tsutsugamushi*.

Cell structure *O. tsutsugamushi* is stained by a modification of the Giménez procedure used for the other rickettsiae; this modified procedure requires preliminary destaining with ferric nitrate and counterstaining with fast green. This species is also satisfactorily stained by Giemsa stain after Carnoy's fixation.

In ultrathin sections, *O. tsutsugamushi* is surrounded by a cytoplasmic membrane and a cell wall, with a clear periplasmic space between the cytoplasmic membrane and cell wall. In the cytoplasm, the electron-dense ribosome-rich area and the less dense network area with DNA fibers are distinctive. *Orientia* possess an unusual Gram-negative cell wall that lacks lipopolysaccharide and peptidoglycan. The outer leaflet of the cell wall is considerably thicker than the inner leaflet, while the opposite is true of *Rickettsia* (Fig. BXII.α.36, F and G) (Silverman and Wisseman, 1978). Possibly as a result, *O. tsutsugamushi* manifests much greater tenacity of adherence to host cell components, which renders purification much more difficult. In contrast to *Rickettsia*, *O. tsutsugamushi* does not have an electron-lucent zone.

Cultivation *O. tsutsugamushi* is cultivated well in the yolk sac of 5–7 d old chicken embryos if the inoculum is relatively large

(10^4 – 10^6 viable cells per egg) and if the rickettsiae are harvested just before the death of the embryos. It is also cultivated well in various cell lines, including HeLa, BHK, Vero, and L929 cells. *O. tsutsugamushi* produces small plaques on cell monolayers after 11–17 d of incubation. CO₂ enrichment is not necessary for intracellular growth (Kopmans-Gargantiel and Wisseman, 1981).

Mice are highly sensitive to infection with *O. tsutsugamushi*. Mice have been used for primary isolation and passage of orientiae. Mice are more sensitive to orientiae by intraperitoneal inoculation than by subcutaneous inoculation.

Multiplication cycle in host cells Orientiae reside free in the cytosol of the host cell. They enter the host cell by attachment and induce phagocytosis. The adhesin has not been identified, but host cell surface heparan sulfate glucosaminoglycan plays a role as a receptor (Ihn et al., 2000). To escape the phagosome, orientiae have to be metabolically active (Rikihisa and Ito, 1982). Movement of orientiae within the cytoplasm to the perinuclear microtubule organizing center, where they replicate, is mediated by microtubules through interaction with dynein—the minus end-directed microtubule associated motor protein—rather than via F-actin tails (Kim et al., 2001). In mouse peritoneal mesothelial cells, *O. tsutsugamushi* multiplies in the cytoplasm, moves to the cell periphery, and separates from the cell surface surrounded by a host cell membrane (Tsuruhara et al., 1982) (Fig. BXII.α.38). Orientiae that are enveloped by the host membrane enter other mesothelial cells, apparently by a phagocytic mechanism. The organisms escape from the phagocytic vacuole as the vacuole membrane and host cell membrane coat disintegrate (Ewing et al., 1978). Cells heavily infected with orientiae undergo apoptotic cell death in association with decreased content of focal adhesion kinase and paxillin, decreased actin stress fiber polymerization, and decreased expression of anti-apoptotic Bcl-2 (Kee et al., 1999). *O. tsutsugamushi* achieves a high intracytoplasmic density, especially in the perinuclear region, and only rarely invades the nucleus.

Nutrition and metabolism There is little information about the nutrition and metabolism of *O. tsutsugamushi*, a topic that has been neglected in the past 20–30 years. *O. tsutsugamushi*-infected cells incorporate ¹⁴C labeled amino acids and adenine at a much higher level than uninfected cells during the period from 3–6 days postinoculation (Weiss et al., 1973). *O. tsutsugamushi* incorporates radiolabeled proline into rickettsial protein (Tamura et al., 1982). In contrast to *Rickettsia*, orientiae do not require CO₂ for growth (Kopmans-Gargantiel and Wisseman, 1981).

Antigenic structure The major proteins of *O. tsutsugamushi* have the following sizes: 110, 80, 70, 60, 56, 47, 42, 35, 28, and 25 kDa (Tamura et al., 1985). Except for the 70- and 60-kDa proteins, all of these are surface proteins. The 56, 28, and 25 kDa proteins are heat labile; they migrate at molecular sizes of 43, 25, and 21 kDa, respectively (Urakami et al., 1986) when they are not heat denatured. The most abundant antigens of *O. tsutsugamushi* are the 60-kDa heat shock protein and the 56-kDa surface protein. The 60-kDa protein is the homolog of the GroEL protein family (Stover et al., 1990). Genetic analysis of the DNA sequence of *groESL* of isolates from several geographic foci has revealed a limited number of variants within each focus and a diversity of strains from focus to focus. In spite of its cytoplasmic location, the 60-kDa protein stimulates antibodies in the sera of some acutely ill patients (Ohashi et al., 1988). The 56-kDa protein varies among geographic isolates of *O. tsutsugamushi* and thus is

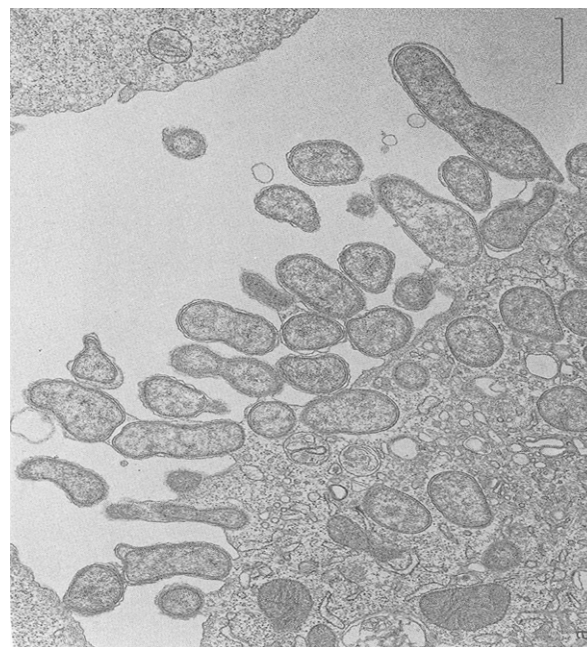


FIGURE BXII.α.38. Electron photomicrograph of *Orientia tsutsugamushi* budding from a host cell at 72 h post infection. Photograph courtesy of A. Tamura and H. Urakami.

called type-specific antigen (TSA; Ohashi et al., 1992). The TSAs of six *Orientia* antigenic variants range from 55,308 to 56,745-daltons with 521–532 amino acids. TSA has the feature of trans-membrane proteins with alternating hydrophobic and hydrophilic regions. Analysis of the TSAs of *Orientia* variants has revealed four variable domains with spans of 16–40 amino acids. The variable domains are located in the hydrophilic regions of the molecule that are likely surface-exposed and show different amino acid sequences among the strains (Ohashi et al., 1992). Early studies of *Orientia* isolates demonstrated three antigenic types: Gilliam, Karp, and Kato. Phylogenetic analyses based on homologies of 56-kDa type-specific antigen genes have classified *Orientia* isolates from China, Korea, Japan, and southeast Asia into seven genotypes designated Gilliam, Karp, Kato, Kawasaki, Kuroki, Shimokoshi and LX-1. All isolates originating in southeast Asia—including the prototype Gilliam and Karp strains isolated in Burma and New Guinea, respectively—are distantly located in the phylogenetic tree from the isolates originating in Japan, Korea, and China; this finding indicates that strains of *O. tsutsugamushi* distributed in northeastern and southeastern Asia are of different types (Enatsu et al., 1999). The antigenic variability of *Orientia* poses a challenge for vaccine design. Immunization with *Orientia* confers relatively strong protection that lasts only 1–3 years against challenge by the homologous strain, whereas protection is very weak and short-lived against heterologous strains (Seong et al., 2001).

The mol% G+C of the DNA of *Orientia* strains is similar to that of TG rickettsiae but lower than that of SFG rickettsiae. The genome of *O. tsutsugamushi* is 1.1 – 1.5×10^9 Da and consists of 2,400–2,700 kbp, as determined by pulse field gel electrophoresis. The genome of *Orientia* is larger than that of *Rickettsia*. The 16S rRNA gene sequence similarity among the antigenic variants of *O. tsutsugamushi*, including the Gilliam, Kato, Karp, Kawasaki, Kuroki, and Shiimokoshi strains, is $\geq 98.5\%$.

When injected intraperitoneally into mice, virulent strains of *O. tsutsugamushi* cause peritonitis, splenomegaly and death in 10–24 days. However, strains can vary greatly in virulence. The Karp strain, for example, is more virulent than the Gilliam strain for most outbred mice. Certain inbred mouse strains are highly resistant to the Gilliam strain; this resistance is controlled by a single, autosomal, dominant gene and does not involve susceptibility to the Karp strain (Groves et al., 1980).

Ecology *Orientia tsutsugamushi* is maintained by transovarian transmission in its various trombiculid mite hosts. Scrub typhus cases occur when larval mites (chiggers) encounter humans as a source of a tissue fluid meal taken from the skin. Each mite host species has its own geographic distribution and seasonal activity pattern that determine the occurrence of scrub typhus (Audy, 1968). Because mites feed on their host only once, the role of the rodents in the maintenance of the natural cycle of rickettsial infection appears even more remote than in the case of the spotted fever rickettsiae. Although wild rats become infected and chiggers acquire rickettsiae when feeding on rats, the chiggers do not transmit the organisms to the next generation (Traub and Wisseman, 1974). *Orientia* requires no other host than the mite in its life cycle.

Orientia tsutsugamushi is encountered in an area of the Orient that extends from India and Pakistan in the west to Japan, the northern portions of Australia, and the intervening islands in the Pacific Ocean in the east and includes southeastern Siberia, Korea, southeast Asia, southern China, the Philippines and Indonesia. These rickettsiae are usually found in circumscribed foci or “ecological islands,” which have the proper vegetation and proper concentration of mites and their wild rat hosts. The habitats are usually characterized by the presence of changing ecological conditions, wrought by man or nature, and expressed by transitional types of vegetation (Traub and Wisseman, 1974).

The mite most commonly associated with scrub typhus is *Lep-totrombidium deliense*, but several other trombiculid mites, including *L. fletcheri*, *L. akamushi*, *L. arenicola*, and *L. scutellare*, have been shown to be naturally infected and to transmit the rickettsia transovarially. Shortly after its emergence from the egg, the six-legged larva, or chigger, either remains in the soil or travels upward a few cm on debris or dead vegetation until it can attach and burrow into the skin of any animal it happens to contact. Following a meal of tissue juices, it returns to the soil to resume a free-living existence. The vertebrates most commonly infected are rodents of the genus *Rattus*, although isolations from temperate zone rodents, including *Apodemus* and *Microtus*, have been reported. The wide dissemination of *O. tsutsugamushi* on islands that are separated from each other and from the mainland by large bodies of water can best be explained by assuming that migrant birds play a role in the transport of the chiggers (Traub and Wisseman, 1974).

ENRICHMENT AND ISOLATION PROCEDURES

Orientia tsutsugamushi is isolated by inoculation of mice, cell culture, or embryonated chicken eggs, as described above. *O. tsutsugamushi* can be identified by polymerase chain reaction amplification of the DNA of a species-specific gene, DNA sequencing of genes that are available in sequence databases, and reactivity with species-specific antibodies.

The cell wall of *Orientia* is fragile and is easily disrupted by mechanical treatment, such as strong homogenization and osmotic shock. *Orientiae* are also very adherent to one another, and it is difficult to resuspend them once they are pelleted. Renografin density gradient centrifugation is not suitable for the purification of *orientiae*. Percoll density gradient centrifugation is the choice for their purification because Percoll has no osmotic effect, and the purification procedure does not involve the pelleting of the organisms until the final stage (Tamura et al., 1982).

Orientia-infected cells are harvested by centrifugation at $300 \times g$ for 10 min and suspended in 1% of the original volume in buffer containing 0.033 M Tris hydrochloride, pH 7.4, and 0.25 M sucrose (TS). The cells are homogenized with a tissue homogenizer with 20–30 strokes. The homogenate is centrifuged at $200 \times g$ for 10 min. The supernatant is mixed with 40% Percoll in TS buffer and centrifuged at $25,000 \times g$ for 60 min. Two bands are formed in the centrifuge tube. The upper band (near the top) consists of cell debris and is removed by suction. The lower band (near the bottom) consists of *orientiae* and is collected with a capillary pipette. The *orientiae* are washed by centrifugation at $6,000 \times g$ for 20 min in TS buffer.

MAINTENANCE PROCEDURES

Stocks of *O. tsutsugamushi* are usually preserved frozen at -70° to -80° or in the vapor phase of liquid nitrogen. They may also be preserved in a lyophilized state. The diluent most frequently used is SPG (0.22 M sucrose, 0.01 M potassium phosphate (pH 7.0) and 0.005 M potassium glutamate) (Bovarnick et al., 1950).

DIFFERENTIATION OF THE GENUS *ORIENTIA* FROM OTHER GENERA

Orientiae grow free in the cytoplasm or nucleus as compared to *Anaplasma*, *Ehrlichia* and *Coxiella*, which grow in the cytoplasm within a vacuole. The characteristics for differentiation between *Rickettsia* and *Orientia* are summarized in Table BXII.α.32 in the chapter on the genus *Rickettsia*.

TAXONOMIC COMMENTS

O. tsutsugamushi, formerly called *Rickettsia tsutsugamushi*, was removed from the genus *Rickettsia* based on the phylogenetic differences between the two genera (Tamura et al., 1995). Phylogenetic analysis of 16S rRNA gene sequences reveals that *O. tsutsugamushi* is located apart from the *Rickettsia* species with a similarity value of 90.2–90.6% (Tamura et al., 1995) (Fig. BXII.α.35).

List of species of the genus *Orientia*

1. ***Orientia tsutsugamushi*** (Hayashi 1920) Tamura, Ohashi, Urakami and Miyamura 1995, 590 (*Rickettsia tsutsugamushi* (Hayashi 1920) Ogata 1931, 252; “*Theileria tsutsugamushi*” Hayashi 1920, 63.)
tsu.tsu.ga.mu.shi. M.L. n. *tsutsugamushi* popular name of the disease caused by this species, generally interpreted to mean mite disease.

The description of the species is the same as that of the genus.

The mol% G + C of the DNA is: 28.1–30.5 (HPLC).

Type strain: Karp, ATCC VR-150.

GenBank accession number (16S rRNA): D38623, U17257.

The type strain is that of Derrick and Brown (1949). Reference strains include Gilliam, ATCC VR-312 (Bennett et al., 1949), and Kato, ATCC VR-609 (Shishido et al., 1958).

Family II. **Anaplasmataceae** Philip 1957, 980^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2156

J. STEPHEN DUMLER, YASUKO RIKIHISA AND GREGORY A. DASCH

A.na.plas.ma.ta'ce.ae. M.L. *Anaplasma* type genus of the family; *-aceae* ending to denote a family; M.L. fem. pl. n. *Anaplasmataceae* type genus of the family.

Rickettsial organisms pathogenic for certain mammals, including man, and birds; non-pathogenic for some arthropods, insects, and helminths. **The predominant mammalian host cells are of bone marrow or hematopoietic origin,** including erythrocytes, monocytes or macrophages, neutrophils, and platelets. Also may grow within tick or other invertebrate cells. Members of the family share a high degree of nucleotide sequence similarity in regard to 16S rDNA and to the *groESL* operon. The **organisms grow within a cytoplasmic vacuole, but not in the nucleus;** they appear as compact to loose inclusions containing as few as one to many

individual organisms. Infected cells may contain more than one inclusion per cell. The mulberry-like appearance of the inclusions has led to the use of the term "morulae" for them. **Organisms may have two distinct morphological forms: dense-core and reticulate bodies.** Gram negative. Nonmotile. **Certain species are adapted to existence in ticks.** Some differential characteristics of the genera of *Anaplasmataceae* are shown in Table BXII.α.33.

Type genus: Anaplasma Theiler 1910, 7 emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157.

TABLE BXII.α.33. Key to the genera of the family *Anaplasmataceae*.

	<i>Anaplasma</i>	<i>Ehrlichia</i>	<i>Neorickettsia</i>	<i>Wolbachia</i>
Host cell infected	Erythrocytes, granulocytes	Mononuclear phagocytes, granulocytes	Mononuclear phagocytes, trematode cells	Arthropod ovaries
Ultrastructural morphology	Multiple bacteria in single vacuole; no intravacuolar fibrillar matrix; vacuoles do not contact mitochondria and endoplasmic reticulum	Multiple bacteria in single vacuole; intravacuolar fibrils present; vacuoles contact mitochondria and endoplasmic reticulum	Small clusters or individuals cells within vacuoles; vacuoles divide with bacterial division	Single bacteria within vacuoles; vacuoles contact endoplasmic reticulum
Mol% G + C of the DNA	43–56	32–46	30–43	Not known

Genus I. *Anaplasma* Theiler 1910, 7^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157

J. STEPHEN DUMLER, YASUKO RIKIHISA AND GREGORY A. DASCH

A.na.plas'ma. Gr. pref. *an* without; Gr. n. *plasma* anything formed or molded; M.L. neut. n. *Anaplasma* a thing without form.

Small, often pleomorphic, coccoid to ellipsoidal cells 0.3–0.4 μm in diameter found in cytoplasmic vacuoles in mammalian host cells, often in inclusion bodies (morulae) in mature or immature hematopoietic cells, in peripheral blood, or in tissues, usually organs containing mononuclear phagocytes (spleen, liver, bone marrow) of mammalian hosts. Morulae are suspended in a non-fibrillar matrix. Two morphologic forms: larger reticulate cells and smaller "dense core" forms. In blood smears stained by Romanowsky methods, the organisms appear as dense, homogeneous, bluish-purple, round inclusions 0.3–2.5 μm in diameter. Gram negative. Nonmotile. No spores or resistant stages. **Ticks are the only known biological vectors;** mechanical vectors include biting flies or other fomites. The organisms can be propagated in tick spp. Some anaplasmas can be propagated in tick cell lines or mammalian cells of hematopoietic origin. **Cause disease in canids, humans, and ruminants.** Variably pathogenic in cattle, goats, sheep, deer, horses, and rodents. Placed in the *Alpha-proteobacteria* by **16S ribosomal RNA sequence analysis; organisms possess not less than 96% 16S rRNA gene similarity.** Where studied, these bacteria possess major surface protein genes with a high degree of sequence similarity nontandemly dispersed throughout the chromosome.

The mol% G + C of the DNA is: 43 and 56.

Type species: Anaplasma marginale Theiler 1910, 7.

FURTHER DESCRIPTIVE INFORMATION

Phylogeny By 16S rRNA gene and *groESL* operon sequence analysis, the genus *Anaplasma* forms a clade distinct from *Ehrlichia*, *Wolbachia*, *Neorickettsia*, *Rickettsia*, and *Orientia* in the *Alpha-proteobacteria*, Order *Rickettsiales*. When 16S rRNA gene and *groESL* operon sequences are aligned, all species in the genus are more than 96% and 74% similar, respectively. Where investigated, organisms possess multiple similar genes that encode major surface proteins that vary in molecular size from approximately 36 to 49 kDa. The bacteria are only distantly related to other obligate intracellular bacteria in genera such as *Chlamydia* and *Coxiella*, and unrelated to bacteria lacking a cell wall, such as *Eperythrozoon* and *Haemobartonella*.

Cell morphology *Anaplasma* species stain bluish-purple with Romanowsky methods. They occur in membrane-bound vacuoles in the cytoplasm of cells of hematopoietic origin, forming inclusions that contain variable numbers of organisms (Simpson, 1972, 1974; Hildebrandt et al., 1973) (See Fig. BXII.α.39). Various spe-

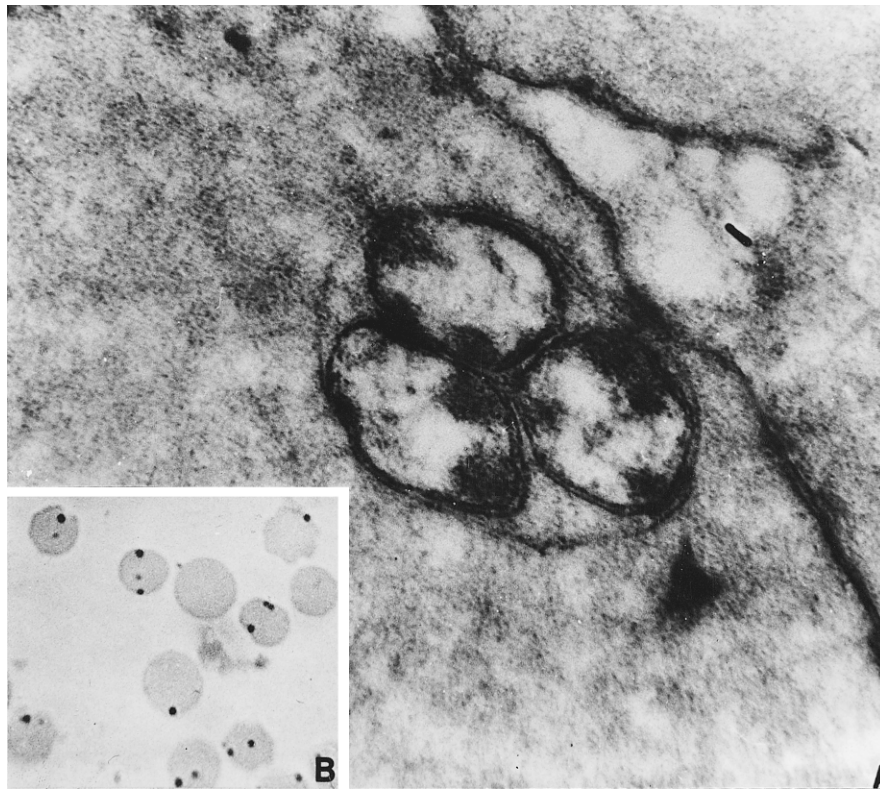


FIGURE BXII.α.39. *Anaplasma marginale*. A, Transmission electron micrograph of an infected erythrocyte. Three individual bacteria bound by cell wall and plasma membrane and lacking intravacuolar fibrils and tubules are shown within the vacuole ($\times 51,000$). B, Intraerythrocytic inclusion bodies stained by a Romanowsky method (Giemsa stain, $\times 815$).

cies infect predominantly erythrocytes, neutrophils, or mononuclear phagocytes *in vivo*. The individual organisms are approximately $0.3\ \mu\text{m}$ in diameter but may vary considerably in size and shape. Inclusions (morulae) range in size up to $4.0\ \mu\text{m}$ in diameter. The organisms are weakly Gram-negative. Anaplasmas are best stained with Romanowsky stains, where the organisms develop a dark blue appearance as opposed to the violet color of the eucaryotic cell nucleus. The organisms stain poorly with the Giménez method but easily with acridine orange.

Cell wall composition Little is known of the components of the cell wall of *Anaplasma* spp. Lipopolysaccharide and lipooligosaccharide have not yet been detected, and it is not known if the cell wall contains peptidoglycan.

Fine structure By electron microscopy, anaplasmas reside within membrane-bound vacuoles that are very early endosomes for *Anaplasma phagocytophilum* (Webster et al., 1998; Mott et al., 1999). The ultrastructure of the cell envelope reveals simple inner and outer leaflets similar to those of Gram-negative bacteria (Popov et al., 1998). The internal structures consisting of chromatin strands and ribosomes are readily visualized. Two distinct morphologic forms may be detected: dense-core forms that contain a relatively dense central or eccentrically-placed condensation of chromatin strands, and reticulate cell forms that contain a homogeneous loose matrix of chromatin strands, among which ribosomes are also spread. Reticulate forms are typically identified *in vivo*, and dense core forms are identified predominantly during *in vitro* propagation. Both forms undergo binary fission, suggesting that a developmental cycle associated with these mor-

phologies is unlikely. *Anaplasma* species may produce an abundant membrane, which on occasion wraps around individual cells or invaginates into the cell. Unlike the genus *Ehrlichia*, *Anaplasma* species lack a fibrillar matrix within the vacuolar space.

Cultural characteristics *A. phagocytophilum* has been successfully propagated in primary human neutrophil cultures and in a variety of human (Goodman et al., 1996) and non-human myeloid or myelogenous lineage cell lines (HL-60, THP-1, M1). *A. phagocytophilum* and *A. marginale* have also been propagated in tick cell tissue culture (Munderloh et al., 1996a, b). Limited propagation of *A. marginale* in erythrocyte culture has also been achieved. No *Anaplasma* species has been cultivated on cell-free media. In cell culture, infection is observed at first with one or few bacteria within the vacuole, previously referred to as an initial body. With continued binary fission of the bacteria, increasingly larger intravacuolar microcolonies are formed, which are called intermediate bodies or morulae, depending upon size. Ultrastructural examination has shown that all vacuoles may contain either reticulate cells, dense-cored cells, or both, and that each of these forms undergoes binary fission; these findings argue against a well-defined intracellular life cycle, as observed for *Chlamydia* spp. Multiple infected vacuoles may exist in a single cell, presumably owing to the lack of fusion of infected endosomes.

For *A. marginale*, recent data suggest that a restricted set of major surface protein genes (*msp2*) may be transcribed during tick infection. However, these results were not corroborated when different strains were examined, which again argues against distinct life cycle-specific bacterial components.

Antigenic structure All *Anaplasma* species contain a multi-gene family encoding major surface proteins that are the immunodominant antigens of the genus. The genes are generally characterized by the presence of highly conserved 5' and 3' sequences that flank a hypervariable core region (Barbet, 1995; Zhi et al., 1998; Viseshakul et al., 2000). One or several of these genes may be transcriptionally active to produce protein antigens of various molecular sizes ranging from 36 to 49 kDa (Asanovich et al., 1997). Other antigenic components may be conserved among species, such as the heat shock proteins (GroESL), or they may be species-specific.

Antibiotic sensitivity All *Anaplasma* species are susceptible to tetracycline antibiotics. Some fluoroquinolone antibiotics, such as enrofloxacin and trovofloxacin, have *in vivo* and *in vitro* efficacy for some species (Guglielmone et al., 1996; Klein et al., 1997). Anaplasmas are not susceptible to β -lactam antibiotics, aminoglycosides, macrolides, chloramphenicol, or sulfa-containing drugs.

Pathogenicity Little is known about the mechanisms by which *Anaplasma* species cause disease. *In vitro*, infected cells undergo both necrosis and apoptosis, and erythrocytes may lyse (Goodman et al., 1996; Waghela et al., 1997). The *in vivo* infection may be associated with febrile hemolytic anemia for *A. marginale*, and with non-specific febrile illness with leukopenia, mild anemia, thrombocytopenia, and mild hepatic injury for *A. phagocytophilum*. While the anemia associated with *A. marginale* may result from direct erythrocyte lysis, it is highly unlikely that the degree of cytopenia observed with *A. phagocytophilum* infections results from direct cytolysis of infected cells (Bakken et al., 1996). "*A. platys*" is associated with febrile cyclical thrombocytopenia associated with the appearance of morulae in platelets in peripheral blood. It is likely that most infections are asymptomatic. Clinically apparent infection may be observed in a range of animals including cattle, goats, sheep, deer, llamas, dogs, horses, and humans.

Anaplasma species gain access to their host cells by adherence to surface components that generally are glycosylated scaffold proteins (McGarey and Allred, 1994; Herron et al., 2000). After adherence, the bacteria are internalized and remain within vacuoles that are early endosomes for *A. phagocytophilum* (Mott et al., 1999). Infection of granulocytic cells *in vitro* results in the secretion of chemokines (Klein et al., 2000), whereas infection of neutrophils *in vitro* results in delayed apoptosis (Yoshiie et al., 2000).

Ecology Ticks are vectors for all *Anaplasma* species that have been studied. *Dermacentor andersoni*, *Boophilus microplus*, *Ixodes scapularis*, *I. pacificus*, and *I. ricinus*, among other ticks, are important biological vectors. Mechanical vectors, such as male ticks and biting flies are also important for transmission of *A. marginale*. *Anaplasma* species are ingested by adult and nymphal stage ixodid ticks when feeding on an infected host. The bacteria are maintained in the tick by transstadial, but not transovarial transmission; thus, emergent larval ticks are not infectious. Domestic and wild ruminants—especially cervids—may be important reservoirs for all species of the genus. Other important reservoirs for *A. phagocytophilum* include small mammals such as mice and other rodents.

ENRICHMENT AND ISOLATION PROCEDURES

Both *A. marginale* and *A. phagocytophilum* have been isolated by inoculation of whole blood in tick cell cultures (Munderloh et

al., 1996a, b), whereas *A. phagocytophilum* is easily isolated in granulocyte cell cultures using whole blood or leukocyte preparations (Goodman et al., 1996). *Anaplasma* species are obligately intracellular bacteria that cannot be isolated by axenic methods. Isolation has also been achieved by inoculation of fresh or frozen blood from infected animals into naïve susceptible animals, such as cows for *A. marginale* and horses or mice for *A. phagocytophilum*.

MAINTENANCE PROCEDURES

A. marginale can be maintained for long periods in tick cell culture, and for shorter periods in erythrocyte cultures (Munderloh et al., 1996b; Waghela et al., 1997). *A. phagocytophilum* can be maintained for long periods (>1 year) by serial propagation in granulocyte cell lines such as HL-60 cells, although *in vitro* passage may alter the propensity of the bacteria to elicit clinical manifestations in susceptible animals (Goodman et al., 1996; Pusterla et al., 2000a). Cultured cells containing the bacteria may be stored in medium containing 30% fetal bovine serum and 10% dimethyl sulfoxide at -80°C for months to years, and in liquid nitrogen for years. Both *A. marginale* and *A. phagocytophilum* can be maintained by serial passage in susceptible animals hosts. Neither *A. bovis* nor "*A. platys*" have been cultivated *in vitro*.

DIFFERENTIATION OF THE GENUS *ANAPLASMA* FROM OTHER GENERA

Anaplasma species may be morphologically difficult to distinguish from *Ehrlichia* and *Neorickettsia* species that grow within bone marrow-derived cells. The genus is separated from related genera by its obligate intracellular growth in bone marrow-derived cells or tick cells, by its growth within a vacuole of the infected host cell, and by finding similarities of >97% in the 16S rRNA gene sequence or >74% in the *groESL* operon sequences compared to sequences of established strains.

TAXONOMIC COMMENTS

Phylogenetic comparisons comprise the most objective comparators and are used as the major tool to evaluate genetic similarities and to establish objective and reproducible taxonomic criteria that cannot be achieved reliably with phenotypic or clinical data. *A. marginale* is the most distinct species in the genus in terms of phenotypic and genotypic characteristics; in fact, some authorities consider this taxon to qualify for a separate genus position.

A. marginale, *A. ovis*, and *A. centrale* are all recognized as valid species in the genus *Anaplasma*. However, the 16S rRNA gene sequences of strains of each, with the exception of a single Japanese strain, are at least 99.1% similar. Thus, it is likely that these represent single species variants, as initially suggested by Theiler (1911). Moreover, the tight clustering of erythrocytic anaplasmas with *A. marginale* is also supported by other genotypic and phenotypic characters, including shared 19, 36, and 105 kDa protein antigens (McGuire et al., 1984; Palmer et al., 1988a; Visser et al., 1992; Palmer et al., 1998). Although *A. caudatum* is proposed and listed as a unique species, some authorities believe that it is a species synonymous with *A. marginale* that represents an artifact of experimental, rather than natural, tick-transmitted infection. A strain of *A. centrale* exists that has 1.8% 16S rRNA gene nucleotide divergence from other *A. centrale* and *A. marginale* strains that were phenotypically characterized, casting significant doubt on morphological methods of taxonomy for this species and genus and suggesting that other erythrocytic *Anaplasma* species exist. Additional study will be required to delineate the precise taxonomic relationships among these closely related bacteria.

The name *Anaplasma* was selected by Dumler et al. (2001) for the emended, newly combined genus because of historical precedence (Theiler, 1910). The exact identity of the previously described "*Cytoecetes microti*" (Tyzzer, 1938) is not certain; however, there is a clear phenotypic similarity to *A. phagocytophilum*. The name "*Cytoecetes*" is not recognized on the Approved Lists of Bacterial Names, but if sufficient data become available that support the separation of *Anaplasma marginale* (and other erythrocytic anaplasmas such as *A. ovis*) from *A. phagocytophilum*, *A. bovis*, and "*A. platys*", the designation "*Cytoecetes*" would have historical precedence and would be validly published.

FURTHER READING

Barbet, A.F. 1995. Recent developments in the molecular biology of anaplasmosis. *Vet. Parasitol.* 57: 43–49.

DIFFERENTIATION OF THE SPECIES OF THE GENUS ANAPLASMA

The preferred method for definitive identification of *Anaplasma* species depends upon sequence analysis of all or part of the 16S rRNA gene or the *groESL* operon or by PCR amplification using species-specific oligonucleotide primers. The full 16S rRNA gene sequence may be identified by many different methods, including commercially available systems such as the MicroSeq[®] Full Gene 16S rDNA Bacterial Sequencing Kit (Applied Biosystems, Foster

Dumler, J.S., A.F. Barbet, C.P.J. Baker, G.A. Dasch, G.H. Palmer, S.C. Ray, Y. Rikihisa and F.R. Rurangirwa. 2001. Reorganization of the genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: Unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Microbiol.* 51: 2145–2165.

Palmer, G.H., W.C. Brown and F.R. Rurangirwa. 2000. Antigenic variation in the persistence and transmission of the ehrlichia *Anaplasma marginale*. *Microbes Infect.* 2: 167–176.

Rikihisa, Y. 1991. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin. Microbiol. Rev.* 4: 286–308.

City, CA). Species-specific primers for *A. marginale*, *A. phagocytophilum* and "*A. platys*" are shown in Table BXII.α.34. Various methods and protocols for diagnostic identification in clinical samples have been described; the diagnostic sensitivity and specificity should be determined by each laboratory performing the clinical assay.

See Table BXII.α.35 for key features of *Anaplasma* species.

List of species of the genus *Anaplasma*

1. *Anaplasma marginale* Theiler 1910, 7^{AL}

margi.na'le. L. n. *margo*, *marginis* edge, margin; M.L. neut. adj. *marginale* marginal, referring to location of the organism within the erythrocytes.

The characteristics are as described for the genus and as listed in Tables BXII.α.35 and BXII.α.36. See also Fig. BXII.α.39.

A. marginale is the causative agent of bovine anaplasmosis, a severe febrile hemolytic anemia of cattle that occurs after tick bites or mechanical transmission by other arthropods. The disease is worldwide in distribution.

A. marginale possesses polymorphous major surface proteins (MSPs), several of which are similar to proteins in *A. phagocytophilum* and some of which are encoded by multi-gene families (Barbet, 1995). A low level, cyclical, persistent infection is established and is accompanied by emergence of antigenic variants in MSP2. MSP1a, MSP1b, and MSP2 are associated with hemagglutinating activity and may represent adhesins of *A. marginale* (McGarey and Allred, 1994). Epitopes of MSP2 and MSP5 are conserved among *A. marginale*, *Anaplasma centrale*, and *Anaplasma ovis*, and both the *msp2* and *msp3* genes of *A. marginale* are present in the genome of *A. ovis*. No tick-specific antigen expression has been proven (Rurangirwa et al., 1999, 2000).

Tetracycline compounds and dithiosemicarbazone inhibit replication and ameliorate clinical manifestations. Penicillin, streptomycin, sulfonamides, and arsenicals are inactive (Barrett et al., 1965; Ristic, 1981).

Since infection results in low-level persistence, natural immunity is incomplete. Immunity that results after elimination of persistently infected state may persist for at least 8 mo. Vaccination using whole intact bacteria or subcomponents has resulted in variable levels of protective immunity (Palmer et al., 1988a; Montenegro-James et al., 1991; Tebele et al., 1991). Cross-protection between *A. marginale* and *A. centrale* has been reported (Palmer et al., 1988b).

The relationship of *A. marginale* to erythrocytic anaplasmas other than *A. centrale* is not certain, although distinct sequences of the 16S rRNA genes have been reported.

A. marginale causes clinical signs with infection in cattle, occasionally sheep or goats, and giraffes (Kuttler, 1984). Inapparent infection may occur in zebu, water buffalo (*Babalis babalis*), bison (*Bison bison*), African antelopes, black wildebeest (*Connochaetes gnou*), blesbuck (*Damaliscus albi-frons*), and duiker (*Sylvicapra grimmii grimmii*), American deer (southern black-tailed, Rocky Mountain mule deer, Virginia white-tailed), pronghorn (*Antilocapra americana americana*), elk, bighorn sheep (*Ovis canadensis canadensis*), and camel (*Camelus bactrianus*). *A. marginale* has not been demonstrated to be infectious for rodents or other small mammals. Persistent infection leading to a carrier "reservoir" state is best documented for cattle; other domestic and wild ungulates and ruminants may also play a role (Kuttler, 1984). Vectors for *A. marginale* include *Dermacentor andersoni* ticks in North America and *Boophilus microplus* ticks in Africa. Transstadial but not transovarial transmission occurs (Stich et al., 1989); thus, the predominant mechanism for natural maintenance involves a mammalian reservoir that is expanded by tick vectors and mechanical or fomite vectors.

Isolation is best achieved by serial passage of blood from infected to susceptible animals. The bacterium may be maintained in stable form when frozen ($\leq -70^{\circ}\text{C}$) in anticoagulated blood containing dimethyl sulfoxide; such preparations can be later transfused into susceptible hosts. The bacteria may also be propagated *in vitro* by cultivation in IDE8 tick cells (Munderloh et al., 1996b). Isolated bacteria may be identified by polymerase chain reaction amplification of conserved *A. marginale* genes, by hybridization of bacterial DNA with *A. marginale* DNA probes, or with *A. marginale* monoclonal or polyclonal antibodies (McGuire et al., 1984; Barbet, 1995; Torioni et al., 1998).

A. marginale persists at low levels in the blood of infected

TABLE BXII.α.34. Species-specific oligonucleotide primers for PCR identification of *Anaplasma* species.

<i>Anaplasma</i> species	Targeted gene	Forward primer	Reverse primer	Hybridization probe
<i>A. marginale</i> ^a	<i>msp1</i>	5'-GTATGGCAGTAGTCTTGGGATCA-3'	5'-CAGCAGCAGCAAGACGTTCA-3'	
<i>A. phagocytophilum</i> ^b	16S rRNA	5'-AAGCGATTAGGCTTATAGCTTCT-3'	5'-TTCCGTTAAGAAAGGATCTAATCTCC-3'	
<i>A. platys</i> ^{cc}	16S rRNA	5'-TGTCGTAGCTTGCTATG-3'	5'-CGTTTGTCTCTGTGTG-3'	5'-GAAGATAATGACGGTATACCC-3'

^aFrom Stüch et al., 1993.^bFrom Edelman and Dumler, 1996.^cFrom Chang and Pan, 1996.

cattle (Torioni et al., 1998). During the initial infection, antigenic variants emerge because of expression of new MSP2 genes, perhaps by recombinational events at the level of the chromosome (French et al., 1998). The nucleic acid sequences of the multigenes in the genome of a single *A. marginale* isolate may be as little as 25% identical. Several of the MSPs of *A. marginale* form complexes within the membrane of the bacterium.

A. marginale is the most phylogenetically distinct species in the genus *Anaplasma*, differing from other species by between 2.3 and 3.5% identity in the 16S rRNA gene sequence and by 25% in the *groESL* operon sequence.

The mol% G + C of the DNA is: 56 (spectral analysis; Alleman et al., 1992).

Type strain: no culture isolated.

GenBank accession number (16S rRNA): M60313.

2. **Anaplasma bovis** (Donatien and Lestoquard 1936) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2158^{VP} (*Rickettsia bovis* Donatien and Lestoquard 1936, 1061; "*Ehrlichia bovis*" Moshkovski 1945, 18.) *bo'vis*. L. n. *bos* the ox; L. gen. n. *bovis* of the ox.

The characteristics are as described for the genus and as listed in Tables BXII.α.35 and BXII.α.36. Cells are coccoid to ellipsoidal and are often pleomorphic. They infect mononuclear phagocytes, mostly monocytes, of cattle. The morphologic and ultrastructural appearance is not different from other members of *Anaplasma*.

A. bovis is the causative agent of bovine ehrlichiosis of Africa, the Middle East, India, and Sri Lanka, a disease that is clinically characterized by fluctuating fever, lymphadenopathy, depression, and, occasionally, death (Rioche, 1966). Infection is usually inapparent in endemic regions, except when exacerbation occurs during periods of stress, with splenectomy, or with other infections such as rinderpest.

A. bovis has not yet been cultivated *in vitro*, but may be propagated by serial passage in susceptible cattle. Identification may be achieved by sequence analysis of polymerase chain reaction-amplified DNA from animal blood or ticks.

The principal clinical manifestations include fever, anorexia and diarrhea, and—infrequently—involvement of the central nervous system with drowsiness and convulsions. Leukopenia and thrombocytopenia may occur.

A. bovis is transmitted by ticks, including *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, in Africa (Rioche, 1966; Matson, 1967) and possibly by *Amblyomma cajennense* ticks in Guadeloupe and Brazil. *A. bovis*-like 16S rRNA gene sequences have been detected in *Haemaphysalis leporispalustris* and *Ixodes scapularis* ("*dammini*") ticks on Nantucket Island in the northeastern U.S. (Goethert and Telford, 2000). This latter observation was also associated with detection in cottontail rabbits, suggesting the existence of a non-ruminant ecological cycle. *A. bovis* infections have been described in North America, Africa, the Middle East, and Asia (India and Sri Lanka).

The 16S rRNA gene sequences are represented by two submissions in GenBank, one from South Africa and one from North America, which are ≥99.5% identical.

The mol% G + C of the DNA is: not known.

Type strain: no strain isolated.

GenBank accession number (16S rRNA): U03775.

TABLE BXII.α.35. List of diagnostic features of *Anaplasma* species.

	<i>A. bovis</i>	<i>A. marginale</i>	<i>A. phagocytophilum</i>	" <i>A. platys</i> "
Usual host species	Cattle	Cattle	Ruminants, horses, dogs, humans	Dogs
Usual infected host cell type	Monocytes	Erythrocytes	Neutrophils	Platelets
Present in peripheral blood ^a	±	++	++	+
Serological reactions with: ^b				
<i>A. bovis</i>	+++	—	—	?
<i>A. marginale</i>	?	+++	—	?
<i>A. phagocytophilum</i>	?	—	+++	—
" <i>A. platys</i> "	?	?	—	+++
<i>Ehrlichia canis</i>	+	—	+	—

^aSymbols: ±, rarely present or difficult to identify; +, present infrequently; ++, present moderately often.

^bSymbols: —, none; +, weak; +++, strong; ?, unknown or not reported.

TABLE BXII.α.36. Descriptions of the species of the genus *Anaplasma*.

	<i>A. marginale</i>	<i>A. bovis</i>	<i>A. phagocytophilum</i>	" <i>A. platys</i> "
Geographic distribution	Worldwide	Africa, Asia, North America?	Europe, Asia, North America, South America	North America, Europe, Taiwan
Infected host(s) with clinical signs	Cattle, ruminants	Cattle	Ruminants, horses, dogs, humans	Dogs
Reservoir host(s)	Cattle, wild ruminants and cervids (deer, elk, water buffalo, sheep)	Cattle?	Small rodents, wild ruminants and cervids, felids (mountain lions), black bear	Dogs
Vectors	<i>Boophilus microplus</i> , <i>Dermacentor andersoni</i> , mechanical vectors (biting flies, fomites)	<i>Rhipicephalus appendiculatus</i> , <i>Amblyomma variegatum</i> , <i>Amblyomma cajennense</i> , <i>Ixodes scapularis</i> (dammini)?, <i>Haemaphysalis leporispalustris</i>	<i>Ixodes scapularis</i> , <i>I. pacificus</i> , <i>I. ricinus</i> , <i>I. persulcatus</i> , <i>I. spinipalpis</i>	<i>Rhipicephalus sanguineus</i> ?
Host cell	Erythrocytes	Monocytes	Granulocytes (neutrophils, eosinophils, basophils)	Platelets
<i>In vitro</i> cultivation	Tick cells, erythrocytes, endothelial cells	—	Granulocyte cell lines, tick cells, endothelial cells	—

3. ***Anaplasma caudatum*** (Kreier and Ristic 1963) Ristic and Kreier 1984c, 355^{VP} (Effective publication: Ristic and Kreier 1984a, 722) (*Paranaplasma caudatum* Kreier and Ristic 1963, 701)

cau'da.tum. L. neut. n. *cauda* tail; *caudatum* tailed, with a tail.

Anaplasma caudatum is similar to *A. marginale* except that each cell possesses an appendage, usually in the form of a tapering tail, a loop, a disk, or ring, that can only be visualized through immunologic or ultrastructural techniques. By electron microscopy, the tails are not directly attached to the bacterium, but contain some bacterial-specific antigens and are predominantly composed of polymerized F-actin filaments of unknown origin (Kocan et al., 1978; Stich et al., 1997). The exact relationship of *A. caudatum* to *A. marginale* is uncertain.

The mol% G + C of the DNA is: unknown.

Type strain: no culture isolated.

4. ***Anaplasma centrale*** (ex Theiler 1911) Ristic and Kreier 1984c, 355^{VP} (Effective publication: Ristic and Kreier 1984a, 722.)

cen.tra'le. L. neut. adj. *centrale* central, referring to the location of the organism within erythrocytes.

Anaplasma centrale is similar to *A. marginale*, except that the disease produced in cattle is usually mild, and the lo-

cation of the bacterium within the erythrocyte cytoplasm is central and not peripheral.

This species shares many genes and antigens with *A. marginale* and *A. ovis* (Palmer et al., 1988b, 1998; Visser et al., 1992). Cross-protection between *A. marginale* and *A. centrale* has been reported (Abdala et al., 1990; Turton et al., 1998). Strains of *A. centrale* possess both specific and genus antigens; however strains of *A. marginale* differ from each other as much as they do from *A. centrale* (McGuire et al., 1984; Palmer et al., 1988b; Visser et al., 1992).

The exact relationship of *A. centrale* to *A. marginale* is uncertain, but the 16S rRNA genes are at least 99.2% identical.

The mol% G + C of the DNA is: not determined.

Type strain: Israel.

GenBank accession number (16S rRNA): AF309869.

5. ***Anaplasma ovis*** Lestoquard 1924, 784^{AL}
o'vis. L. gen. n. *ovis* of the sheep.

Anaplasma ovis is similar to *A. marginale* except that the hosts with clinical signs are usually restricted to sheep and goats.

This species contains multiple genes that are also present in *A. marginale*, including *msp5* and multiple *msp2* and *msp3* genes (Visser et al., 1992; Palmer et al., 1998).

Cross-protection between *A. marginale* and *A. ovis* has

not been reported, although T cells from *A. marginale* immunized animals proliferate when exposed to *A. ovis* antigens (Brown et al., 1998).

The exact relationship of *A. ovis* to *A. marginale* is uncertain, but the sequences of the 16S rRNA genes are at least 99.6% identical.

The mol% G + C of the DNA is: not determined.

Type strain: Idaho.

GenBank accession number (16S rRNA): AF309865.

6. **Anaplasma phagocytophilum** (Foggie 1951) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2158^{VP} (*Rickettsia phagocytophila* Foggie 1951, 4; *Ehrlichia phagocytophila* Philip 1962, 42.)

pha.go.cy.to'phi.lum. Gr. inf. *phagein* to eat, devour; Gr. n. *kytos* a vessel, enclosure; Gr. inf. *philein* to love; M.L. adj. *phagocytophilum* fond of devouring cells (in microbiology, attractive to phagocytes).

The characteristics are as described for the genus and as listed in Tables BXII.α.35 and BXII.α.36. See also Figs. BXII.α.40 and BXII.α.41. Cell morphology is as described

for the genus. Morulae can be up to 6 μm in diameter (Popov et al., 1998). Dense-core and reticulate cells occur together in vacuoles; both forms divide by binary fission. There is no fibrillar matrix; the vacuolar space may contain empty vesicles. The bacterial cytoplasmic membrane may protrude into the periplasmic space or invaginate into the interior of the cell.

A. phagocytophilum causes tick-borne fever of ruminants, mainly in Western Europe (Gordon et al., 1932; Hudson, 1950; Foggie, 1951). Strains previously known as *Ehrlichia equi* and HGE agent cause equine granulocytic ehrlichiosis (Gribble, 1969; Stannard et al., 1969; Madigan and Gribble, 1987), a form of canine granulocytic ehrlichiosis (Greig et al., 1996; Pusterla et al., 1997), and human granulocytic ehrlichiosis in northern Europe and the Western Hemisphere (Bakken et al., 1994; Chen et al., 1994; Goodman et al., 1996). Serological evidence indicates that asymptomatic infection occurs in humans and animals. Clinical signs in humans and animals include fever accompanied by leukopenia and thrombocytopenia; opportunistic infections may occur in humans and animals (Walker and Dum-

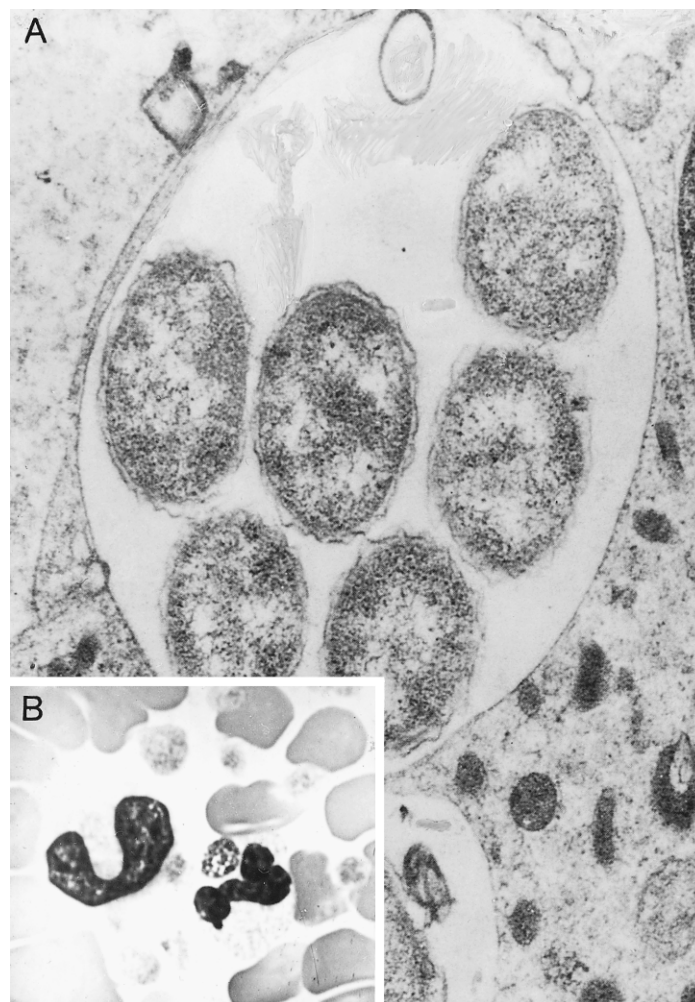


FIGURE BXII.α.40. *Anaplasma phagocytophilum* A, Transmission electron micrograph of an infected equine blood granulocyte with an intracytoplasmic inclusion body (morula). Several single bacteria bound by a rippled cell wall and plasma membrane are evident (× 51,000). (Reproduced by permission from D.M. Sells et al., *Infection and Immunity*, 13: 273–280, 1976, ©American Society for Microbiology, Washington, D.C.). B, An intragranulocytic inclusion body stained by a Romanowsky method (Giemsa stain, × 1,020) (Courtesy of Cynthia Holland).

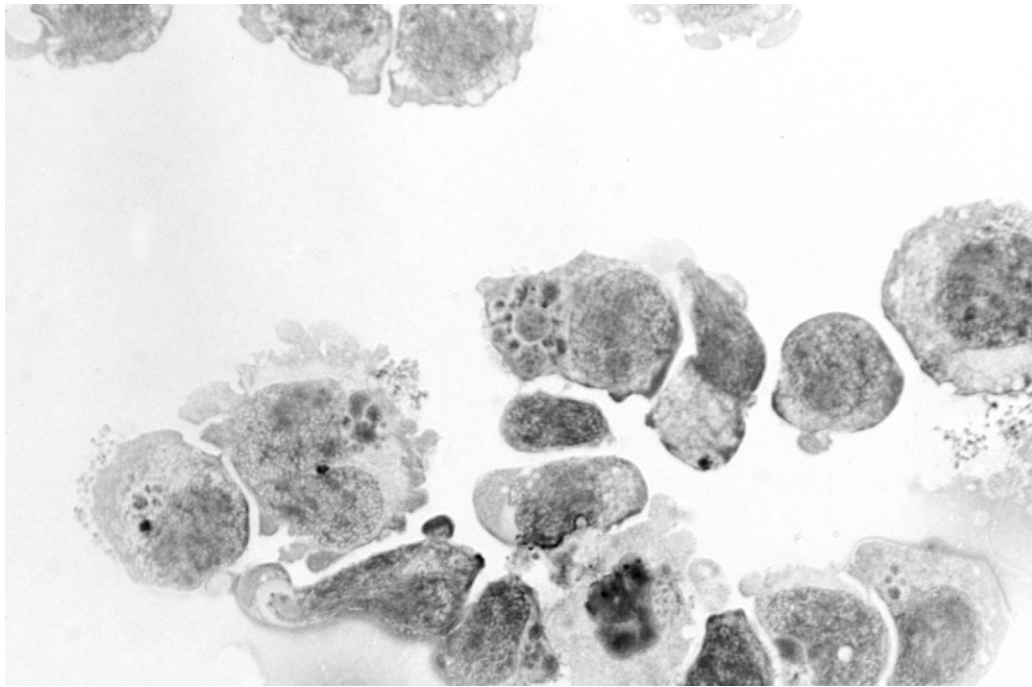


FIGURE BXII.α.41. *Anaplasma phagocytophilum* intracytoplasmic inclusions (morulae) that contain many individual bacteria in cultured human HL-60 promyelocytic leukemia cells, stained with a Romanowsky stain (LeukoStat stain, $\times 360$).

ler, 1996). Other signs include depression and anorexia in horses and dogs and limb edema and ataxia in horses. Humans may suffer liver damage (Aguero-Rosenfeld et al., 1996; Bakken et al., 1996). The organism is widely distributed geographically.

Tick vectors include species of the *Ixodes persulcatus* complex (MacLeod and Gordon, 1933; Foggie, 1951; Richter et al., 1996; Telford et al., 1996). Organisms are maintained in ticks by transstadial but not transovarial transmission. Important reservoirs include rodents (*Peromyscus leucopus*) and potentially large mammals such as cattle and wild ruminants, including cervids and deer.

All isolates cross-react serologically and share antigens with *Ehrlichia canis*, *E. chaffeensis*, and *E. ruminantium*. Major antigens are 42–49 kDa outer membrane proteins encoded by a multigene family (Dumler et al., 1995; Asanovich et al., 1997; Zhi et al., 1997, 1998; Murphy et al., 1998). The primary structures of these antigens are similar to corresponding antigens of *A. marginale*, *E. ruminantium*, *E. canis*, *E. chaffeensis*, and *Wolbachia*.

A. phagocytophilum is susceptible to low concentrations of tetracycline antibiotics, rifamycins, and some fluoroquinolones such as trovafloxacin and resistant to moderate or high concentrations of β -lactams, aminoglycosides, macrolides, and chloramphenicol (Klein et al., 1997).

A. phagocytophilum may be isolated from infected animals by passage of fresh or dimethyl sulfoxide-treated frozen anticoagulated blood specimens or by *in vitro* cultivation in granulocyte cultures, such as HL-60 cells, or in tick cells such as IDE8 cells. Infected cells may be stored for several months at -70°C or for years in liquid N_2 if highly infected. The storage medium is RPMI 1640 medium supplemented with 10% dimethyl sulfoxide. Identification is best achieved

by polymerase chain reaction amplification of *A. phagocytophilum*-specific gene targets or by immunocytochemical identification using specific polyclonal or monoclonal antibodies. The genome size is approximately 1500 kbp (Rydikina et al., 1999).

Within the species, *A. phagocytophilum* 16S rRNA gene sequences exhibit $>99.5\%$ identity, and *groESL* sequences exhibit $\geq 99.0\%$ identity.

The mol% G + C of the DNA is: 43.8 (by gene sampling).

Type strain: Webster.

GenBank accession number (16S rRNA): U02521.

7. “**Anaplasma platys**” (French and Harvey 1983) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159 (“**Ehrlichia platys**” French and Harvey 1983, 2410.)

pla'tys. Gr. adj. *platys* flat, the word from which platelet is derived.*

The characteristics are as described for the genus and as listed in Tables BXII.α.35 and BXII.α.36. The cells are coccoid to ellipsoidal, often pleomorphic, and infect platelets of dogs. Except for the presence in platelets, the morphologic and ultrastructural appearance is not different from that of other members of *Anaplasma* (Mathew et al., 1997).

This species is the causative agent of infectious cyclic thrombocytopenia of dogs, a disease that results in decreasingly severe episodes of fever and thrombocytopenia. The bacterium may also cause inapparent infection in rumi-

*Editorial Note: The name of this species has not been validly published, because the etymology of *platys* was missing from the descriptions.

nants (Allsopp et al., 1997). The principal clinical manifestation is thrombocytopenia in the absence of fever and clinical signs. Hemorrhage occurs rarely if associated with surgery or accidents.

The organisms are likely transmitted by ticks, perhaps including *Rhipicephalus sanguineus* or *Amblyomma* spp. The observation of infection in sheep and impalas suggests a potential role for ruminants as reservoirs (Allsopp et al., 1997; Du Plessis et al., 1997).

This species has not yet been cultivated *in vitro*, but it can be propagated by serial intravenous passage in susceptible dogs. Infected blood can be stored in liquid N₂ when diluted 1:2 in phosphate buffered saline containing a final concentration of 7.5% glycerol.

Identification is best achieved by polymerase chain reaction amplification of "A. platys"-specific or white-tailed deer *Ehrlichia*-specific gene targets (Little et al., 1997; Mathew et al., 1997) or by immunocytochemical identification using specific polyclonal antibodies (Simpson and Gaunt, 1991).

"A. platys" is serologically distinct from *Ehrlichia canis*. The relapsing nature of the infection is similar to that seen in bovine anaplasmosis and may similarly be associated with changes in surface protein expression.

The 16S rRNA gene sequences among three strains are ≥99.5% identical, including one strain originating from South African sheep.

16S rRNA gene sequences that have been amplified from the blood of white-tailed deer and an *Amblyomma ameri-*

canum tick in North America are most similar to those of "A. platys", with a nucleic acid sequence identity between 96.5 and 98.1% (Dawson et al., 1996; Brandsma et al., 1999). The 16S rRNA gene sequences indicate that the "white-tailed deer" group forms a reproducible and unique clade that is between 96.9% and 98.6% identical to other members of the *Anaplasma* genus, but is not more than 92.3% identical to any member of the genera *Ehrlichia*, *Wolbachia*, *Neorickettsia*, *Orientia*, or *Rickettsia*. The "white-tailed deer" bacteria have never been morphologically identified or cultivated *in vitro*. "A. platys" has not yet been cultivated *in vitro*.

The mol% G + C of the DNA is: not known.

Deposited strain: no strain isolated.

GenBank accession number (16S rRNA): M82801.

Species Incertae Sedis

1. "**Anaplasma mesaeterum**" Uilenberg, van Vorstenbosch and Perie 1979, 21.

mes.ae.ter' um. L.

The taxonomic relationship of this organism to the genus *Anaplasma* is not known. The organisms were identified in sheep in The Netherlands. The description is similar to that of *A. ovis* except for an increased pathogenicity for sheep and a decreased proportion of peripherally located bacteria in the infected erythrocytes.

Transmission is by either *Ixodes ricinus* or *Haemaphysalis punctata* ticks.

Genus II. *Ehrlichia* Moshkovski 1945, 18^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157

J. STEPHEN DUMLER, YASUKO RIKIHISA AND GREGORY A. DASCH

Ehr.lich'i a. M.L. fem. n. *Ehrlichia* named after Paul Ehrlich, a German bacteriologist.

Coccoid to ellipsoidal cells. Found in cytoplasmic vacuoles in endothelial or hemopoietic mammalian host cells, including macrophages, monocytes, neutrophils, and cells of the bone marrow, liver, spleen, or lymph nodes. Often occur in clusters (morulae). Pleomorphic; occur as **reticulate cells** and **"dense core forms" with condensed cytoplasm** (Popov et al., 1998). Gram negative. Nonmotile. Some species grow in cultured tick cells or in cultured mammalian monocytes, macrophages, or endothelial cells. **Cause disease in ruminants, canids, rodents, and humans. Ticks are the primary vectors and hosts.** Belong to the *Alphaproteobacteria* by 16S rDNA gene sequence analysis; the organisms exhibit ≥ 97.6% 16S rRNA gene sequence identity with one another. Where studied, these bacteria possess surface protein antigen genes that are tandemly arranged in the chromosome and have a high degree of sequence similarity (Ohashi et al., 1998a, b; Reddy et al., 1998; McBride et al., 2000; Yu et al., 2000a).

The mol% G + C of the DNA is: 30–56.

Type species: *Ehrlichia canis* (Donatien and Lestoquard 1935) Moshkovski 1945, 18 (*Rickettsia canis* Donatien and Lestoquard 1935, 419) emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159.

FURTHER DESCRIPTIVE INFORMATION

Phylogeny By 16S rRNA gene and *groESL* operon sequence analysis, the genus *Ehrlichia* forms a clade distinct from *Ana-*

plasma, *Wolbachia*, *Neorickettsia*, *Rickettsia*, and *Orientia* in the *Alphaproteobacteria*, Order *Rickettsiales*. All species in the genus show more than 97.6% and 86.3% similarity in the 16S rRNA gene and *groESL* operon sequences, respectively. Where investigated, the organisms possess multiple similar genes that encode surface protein antigens varying in molecular size from approximately 28–32 kDa (Ohashi et al., 1998a, b; Reddy et al., 1998; McBride et al., 2000; Yu et al., 2000a). The bacteria are only distantly related to other obligate intracellular bacteria in genera such as *Chlamydia* and *Coxiella* and are unrelated to cell-wall-free bacteria such as *Eperythrozoon* and *Haemobartonella*.

Cell morphology Ehrlichiae occur in membrane-bound vacuoles in the cytoplasm of host cells of hematopoietic origin and, for some species, in endothelial cells. The bacteria form inclusions (morulae) that contain variable numbers of organisms (Simpson, 1972, 1974; Hildebrandt et al., 1973) (See Figs. BXII.α.42 and BXII.α.43). The individual organisms are approximately 0.5 μm in diameter, but size may vary considerably. The morulae range in size up to 4.0 μm in diameter. The organisms are weakly Gram negative. Ehrlichiae are best stained with Romanowsky stains, where the organisms develop a dark blue appearance as opposed to the violet color of the host cell nucleus. Organisms stain poorly with the Giménez method but easily with acridine orange.

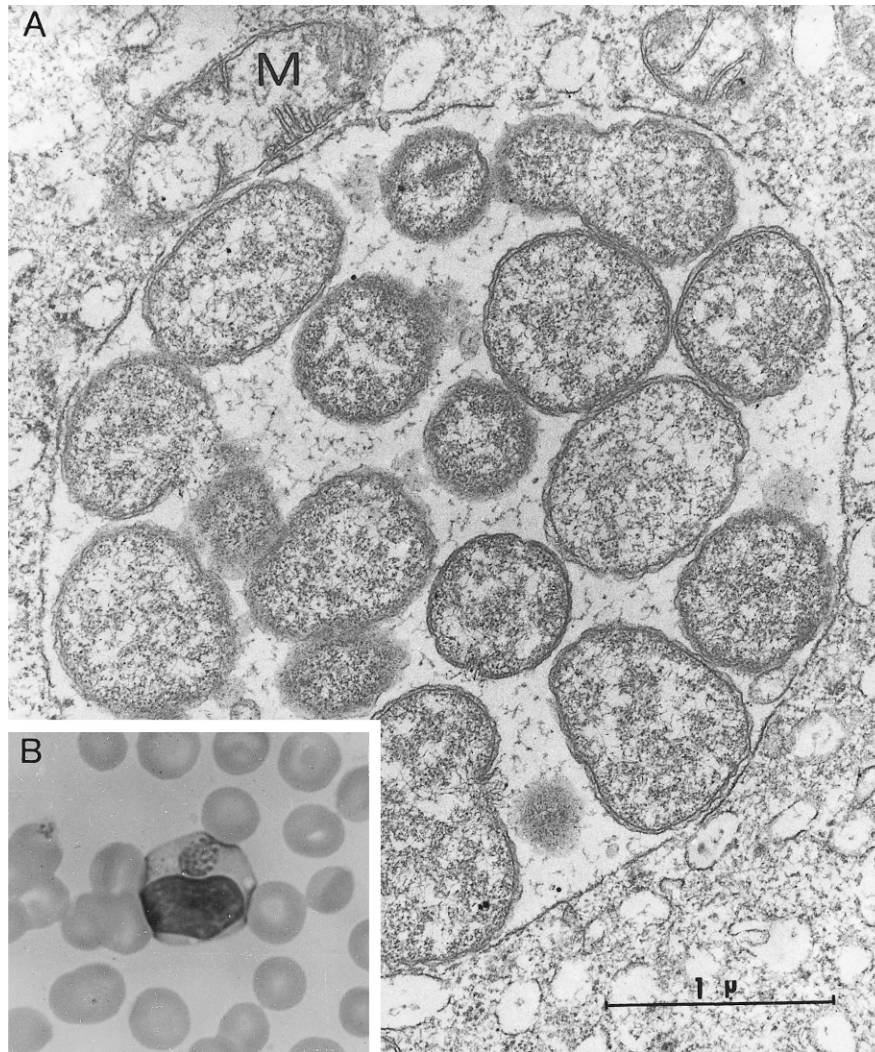


FIGURE BXII.α.42. *Ehrlichia canis*. A, Transmission electron micrograph of an infected canine blood monocyte with an intracytoplasmic inclusion body (morula). Numerous bacteria with distinct plasma membranes and rippled outer cell walls are shown. Note the presence of fibrils and occasional tubules within the intravacuolar, extrabacterial space. M = mitochondrion ($\times 60,000$). (Reproduced with permission from P.K. Hildebrandt et al., *Infection and Immunity*, 7: 265–271, 1973, ©American Society for Microbiology, Washington, D.C. B, An intramonocytic inclusion body stained by a Romanowsky method (Giemsa $\times 680$) (Courtesy of S.A. Ewing, Veterinary Medicine, Oklahoma State University, Stillwater).

Cell wall composition Little is known of the components of the cell wall in *Ehrlichia*. Lipopolysaccharide and lipooligosaccharide have not yet been detected, and it is not known if the cell wall contains peptidoglycan.

Fine structure By electron microscopy, ehrlichiae reside within membrane-bound vacuoles that are early endosomes for *E. chaffeensis* (Mott et al., 1999). The ultrastructure of the cell envelope reveals simple inner and outer leaflets similar to those of Gram-negative bacteria (Popov et al., 1998). Internal structures consisting of chromatin strands and ribosomes are readily visualized. Two distinct morphologic forms may be detected, dense-core forms that contain a relatively dense central or eccentrically located condensation of chromatin strands, and reticulate cell forms that contain a homogeneous loose matrix of chromatin strands among which ribosomes are spread. Reticulate cells are typically identified *in vivo*, and dense core cells are

identified predominantly during *in vitro* propagation. Both forms undergo binary fission, suggesting that a developmental cycle associated with these morphologies is unlikely. *Ehrlichia* species may produce an abundant membrane that on occasion wraps around individual ehrlichiae or invaginates into the ehrlichial cell and occasionally forms tubule and vesicle profiles in the vacuolar space. Unlike the genus *Anaplasma*, *Ehrlichia* species possess a fibrillar matrix within the vacuolar space.

Cultural characteristics *E. canis*, *E. chaffeensis*, *E. ruminantium*, and *E. muris* have been successfully propagated *in vitro*. Most species may be propagated in primary monocyte cultures established from blood or in macrophage/histiocyte cell lines derived from humans, dogs, and mice (e.g. THP-1, DH82, and P388D1, among others). *E. canis* has been successfully cultivated in primary canine monocyte cultures and in canine-mouse hybrid cells (Nyindo et al., 1971; Stephenson and Osterman, 1977; Hemelt

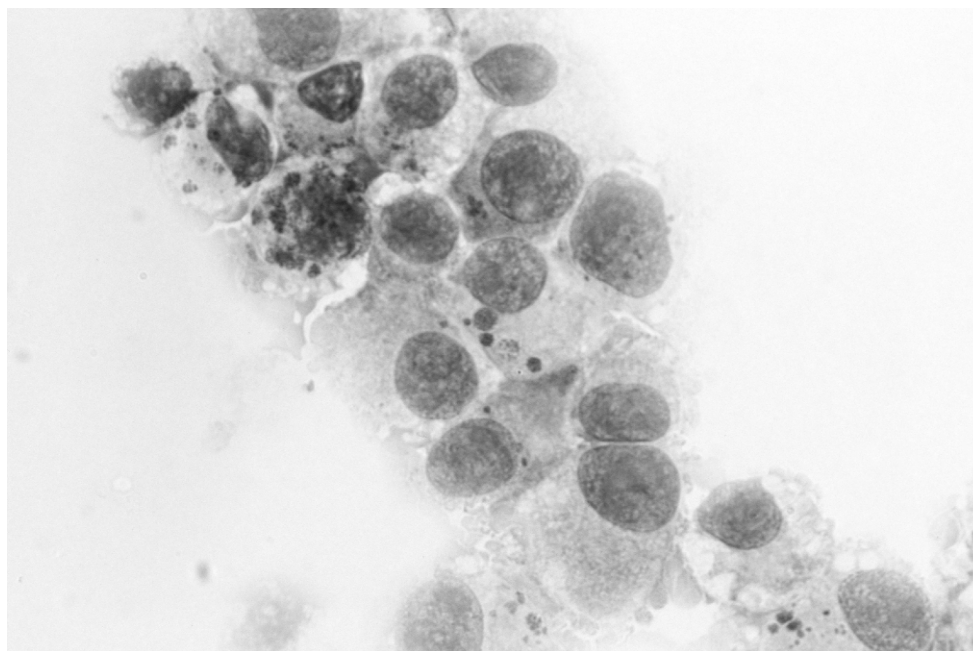


FIGURE BXII.α.43. *Ehrlichia chaffeensis*. Intracytoplasmic inclusions that contain many individual bacteria in cultured canine DH82 histiocytes, stained with a Romanowsky stain (LeukoStat stain, $\times 340$).

et al., 1980; Dawson et al., 1991). *E. chaffeensis* has also been propagated in fibroblast cell lines (Vero, HEL, BGM) and in endothelial cell lines (Dawson et al., 1993; Brouqui et al., 1994; Chen et al., 1995b). *E. ruminantium* is often propagated in primary bovine aortic endothelial cell cultures (Martinez et al., 1993; Yunker, 1995; Perez et al., 1997). *E. canis* has also been propagated in tick cell tissue culture (Ewing et al., 1995). No *Ehrlichia* species has been cultivated on cell-free medium. In cell culture, infection is observed at first with one or few bacteria within the vacuole, previously referred to as an "initial body". With continued binary fission of the bacteria, increasingly larger intravacuolar microcolonies are formed, which are called intermediate bodies or morulae, depending upon size. Ultrastructural examination has shown that all vacuoles may contain either reticulate or dense-cored cells or both and that each of these forms undergoes binary fission; these findings argue against a well-defined intracellular life cycle as observed for *Chlamydia* spp. Multiple infected vacuoles may exist in a single cell, presumably owing to the lack of fusion of infected endosomes.

Antigenic structure All *Ehrlichia* species contain a multigene family that encodes major surface proteins. These proteins are the immunodominant antigens of the genus, and the genes encoding them have a relatively high degree of similarity to corresponding genes in the genera *Anaplasma* and *Wolbachia*. The genes are usually characterized by the presence of several highly conserved sequences that flank several variable sequences. B cell epitopes map to the amino acids encoded by the variable regions, suggesting a role in immune evasion. *In vitro*, from one to many of these genes are transcriptionally active and yield protein antigens of various molecular sizes ranging from 28–32 kDa. Other antigenic components may be conserved, such as the heat shock proteins (GroESL), or they may be species specific.

Antibiotic susceptibility Ehrlichiae are most strongly inhibited during *in vitro* propagation by rifampin and tetracycline anti-

biotics, including tetracycline hydrochloride, oxytetracycline, and doxycycline. Other antibiotics are ineffective, including aminoglycosides, macrolides, fluoroquinolones, chloramphenicol, and β -lactams. Patients with human monocytic ehrlichiosis have a shorter duration of fever and hospitalization when treated with either a tetracycline or chloramphenicol than with other antibiotics.

Pathogenicity The mechanisms by which *Ehrlichia* species cause disease are poorly understood. During *in vitro* propagation, infected mononuclear phagocytes undergo both necrosis and apoptosis. The *in vivo* infection presents as a non-specific febrile illness with leukopenia, mild anemia, thrombocytopenia, and mild hepatic injury for most species. By contrast, *E. canis* infection in some dog breeds results in a persistent infection that terminates with recrudescence months to years later and is often fatal. Infections and ehrlichial loads appear to be more severe in immunocompromised hosts (splenectomized, corticosteroid therapy, HIV infection, transplant recipients, severe combined immunodeficiency); however, under most other circumstances, the degree of cytopenia observed is out of proportion to the quantity of ehrlichiae detected. An exception may occur in *E. ruminantium* infections, where a significant proportion of the pathogenesis may be related directly to vascular injury after endothelial cell infection, particularly in the cerebral microvascular beds. The proportion of infections that result in clinical signs is still not known, but among those with clinical signs, the proportion with severe morbidity may be as high as 10–50%. Clinically evident infection may be observed in a range of animals including cattle, goats, sheep, deer, other domestic and wild ruminants, dogs and other canidae, mice and other small rodents, and humans.

Ehrlichia species gain access to host cells by adherence to surface proteins that are usually glycosylated. The bacteria are then internalized within early endosomes; for *E. chaffeensis* these endosomes selectively accumulate transferrin receptors, effectively

sequestering the infected vacuole into a receptor salvage pathway that precludes lysosome fusion. Infection of macrophages *in vitro* results in low-level secretion of proinflammatory cytokines, whereas binding of immunoglobulin-opsonized *E. chaffeensis* to the macrophage surface results in a high-level release of proinflammatory cytokines.

Ecology Ticks are the primary vectors and hosts for *Ehrlichia* species. Although the microorganisms are passed transstadially in ticks, definite transovarial transmission of identified ehrlichiae has not been demonstrated. The range of potential vertebrate hosts for *Ehrlichia* species is not completely defined owing to a lack of clinical signs in many reservoir hosts, including deer and small mammals. Some mammalian hosts, such as white-tailed deer, some ruminants, and dogs, maintain infectivity for long intervals (months to years) in the absence of clinical signs, whereas other mammals, such as some dog breeds and humans, develop sterile immunity after clinically apparent primary infection. Thus, natural maintenance of ehrlichiae is dependent upon horizontal transmission involving acutely and persistently infected mammals and ticks.

ENRICHMENT AND ISOLATION PROCEDURES

Some species of *Ehrlichia* can be isolated and propagated in cell culture. For isolation, blood is the most appropriate specimen for inoculation. Blood is fractionated into component leukocyte fractions or inoculated directly into appropriate cell cultures, including primary monocyte cultures or cell lines of macrophages (canine DH82, human UL37 or differentiated HL-60, murine P388D1), myelomonocytic cells (human THP-1), fibroblasts (Vero, L929), or endothelial cells. Cultured cells are examined by Romanowsky staining, immunofluorescent staining, or by nucleic acid detection methods to establish the presence of the infectious agent. Primary isolation may require intervals as short as several days or longer than 30 d. Partially purified ehrlichiae may be prepared by density gradient centrifugation using diatrizoate meglumine (Renografin), sucrose, or Percoll gradients.

MAINTENANCE PROCEDURES

E. canis, *E. ewingii*, *E. ruminantium*, and *E. muris* can be serially passed by animal inoculation using blood, spleen, or other tissues. Infectious blood can be stored by suspension with glycerol or dimethyl sulfoxide at 4°C for several days to one week, at -80°C for weeks or months, and in liquid nitrogen for years. Cultured cells containing the bacteria can be stored in media containing 30% fetal bovine serum and 10% dimethyl sulfoxide at -80°C for months to years and in liquid nitrogen for years.

DIFFERENTIATION OF THE GENUS *EHRlichia* FROM OTHER GENERA

Characteristics useful for the differentiation of the genus *Ehrlichia* from the other genera of the family *Anaplasmataceae* are listed in Table BXII.α.33 of the section describing the family. *Ehrlichia* species may be morphologically difficult to distinguish from *Anaplasma* and *Neorickettsia* species that grow within bone marrow-derived cells. By ultrastructural examinations, *Ehrlichia* spp. tend to form large morulae containing many cells suspended in an intravacuolar, extrabacterial fibrillar matrix, whereas *Anaplasma* spp. tend to lack additional intravacuolar structures and form smaller morulae containing low numbers of bacteria. Differen-

tiation is best achieved by using DNA sequence analysis of the 16S rRNA gene or the *groESL* operon.

TAXONOMIC COMMENTS

Historically, *Ehrlichia* species were detected as intracytoplasmic clusters of bacteria (morulae) within peripheral blood leukocytes. An essential characteristic was the presence of the bacteria within membrane-bound vacuoles. Other important characteristics included the host mammalian species infected, the geographic distribution of the infectious agent, and a minor degree of antigenic cross-reactivity among proposed species. Most species were recognized as transmissible by the bite of a tick vector.

With the advent of molecular biological methods, a more accurate understanding of the phylogenetic and potential taxonomic positions of *Ehrlichia* species has emerged. Taxonomic placements in the genus *Ehrlichia* are now largely determined by nucleotide sequence analysis of the 16S rRNA genes and nucleotide and amino acid sequence analyses of the *groESL* operon and GroEL proteins. This information is used in conjunction with an assessment of the degree of antigenic cross-reactivity, the presence of similar outer membrane protein antigens, the tropism for mammalian cell hosts derived from bone marrow precursors, and the range of mammalian and tick hosts.

Members of the genus *Ehrlichia* share greater than 97.6% 16S rRNA gene nucleotide sequence similarity. The branching order established in dendrograms and phylogenetic analyses mimics similar arrangements predicted by the sequence analysis of the *groESL* operon and by antigenic analysis of the GroEL proteins. The specific host cell type, the infected host mammalian species, and the geographic distribution do not correlate with predicted genetic relationships; however, residence within an intracytoplasmic endosome and transmissibility by tick vectors are characteristic of all species. Although clear differences in the types of clinical manifestations are observed in appropriate infected mammalian hosts, the weight of objective genetic, antigenic, and pathogenic features provides unequivocal evidence of an evolutionary linkage. The current genetic taxonomic system has yielded several significant changes from previous methods of classification; these changes include the removal of several species from the genus *Ehrlichia* into the genera *Anaplasma* or *Neorickettsia*, and the integration of the genus *Cowdria* into the genus *Ehrlichia* (Dumler et al., 2001). It is anticipated that whole genome sequences may further still refine the genetic classification and yield new combinations not currently considered.

FURTHER READING

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *EHRLICHIA*

The preferred method for definitive identification of *Ehrlichia* species depends upon sequence analysis of all or part of the 16S rRNA gene or the *groESL* operon or upon PCR amplification using species-specific oligonucleotide primers. The full 16S rRNA gene sequence may be identified by many different methods, including commercially available systems such as the MicroSeq[®] Full Gene 16S rDNA Bacterial Sequencing Kit (Applied Biosys-

tems, Foster City, CA). Species-specific primers for *E. canis*, *E. chaffeensis*, *E. muris*, and *E. ruminantium* are shown in Table BXII.α.37. Various methods and protocols for diagnostic identification in clinical samples have been described; the diagnostic sensitivity and specificity should be determined by each laboratory performing the clinical assay.

List of species of the genus *Ehrlichia*

1. ***Ehrlichia canis*** (Donatien and Lestoquard 1935) Moshkovski 1945, 18^{VP} (*Rickettsia canis* Donatien and Lestoquard 1935, 419) emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159.
ca'nis. M.L. gen. n. *canis* of the dog.

The characteristics are as given for the genus and as listed in Tables BXII.α.38 and BXII.α.39. See also Fig.

BXII.α.42. *E. canis* forms a phylogenetically distinct clade in the genus *Ehrlichia*, differing from other species by 1.3–2.4% in the 16S rRNA gene sequence and by 8.7–14.5% in the *groESL* operon sequence. *E. canis* possesses polymorphic major surface proteins encoded by multigene families similar to those in other *Ehrlichia* species and more distantly related to those in *Anaplasma* and *Wolbachia* species. The

TABLE BXII.α.37. Species-specific oligonucleotide primers for PCR identification of *Ehrlichia* species.

Species	Targeted gene	Forward primer	Reverse primer
<i>E. canis</i>	16S rRNA ^a	5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'	5'-ATAGGGAAGATAATGACGGTACCTATA-3'
<i>E. chaffeensis</i>	16S rRNA ^a	5'-CAATTATTTATAGCCTCTGGTTATAGGA-3'	5'-ATAGGGAAGATAATGACGGTACCTATA-3'
<i>E. ewingii</i>	16S rRNA ^a	5'-CAATTCCTAAATAGTCTCTGACTATTTGA-3'	5'-ATAGGGAAGATAATGACGGTACCTATA-3'
<i>E. muris</i>	16S rRNA ^a	5'-TAGCTACCCATAGCTTTTCTAGCTATAGG-3'	5'-ATAGGGAAGATAATGACGGTACCTATA-3'
<i>E. ruminantium</i>	16S rRNA ^a	5'-CAGTTATTTATAGCTTCGGCTATGAGTATC-3'	5'-ATAGGGAAGATAATGACGGTACCTATA-3'
<i>E. ruminantium</i>	<i>mapI</i> ^b	5'-GATGTAATACAGGAAGAG-3'	5'-CTATTCTTGGTCCATTTC-3'

^aBased upon unique and shared regions as in Anderson et al., 1992b.

^bFrom Kock et al., 1995.

TABLE BXII.α.38. Diagnostic features of *Ehrlichia* species.^a

Characteristic	<i>E. canis</i>	<i>E. chaffeensis</i>	<i>E. ewingii</i>	<i>E. muris</i>	<i>E. ruminantium</i>
Usual host species	Dogs	Dogs, humans, deer	Dogs, humans	Mice	Cattle
Usual infected host cell type	Monocytes	Monocytes	Neutrophils	Monocytes	Endothelial cells, neutrophils
Present in peripheral blood ^a	++	++	+	—	±
Serological reactions with: ^b					
<i>E. canis</i>					++
<i>E. chaffeensis</i>	+++				
<i>E. ewingii</i>	+++	+++			
<i>E. muris</i>	+++	+++	+++		
<i>E. ruminantium</i>	++	++	++	?	
<i>Anaplasma marginale</i>	—	—	—	—	±
<i>Anaplasma phagocytophilum</i>	±	±	±	?	±

^aSymbols: ±, rarely present or difficult to identify; +, present infrequently; ++, present moderately often.

^bSymbols: —, none; +, weak; ++, moderate; +++, strong; ?, unknown or not reported.

TABLE BXII.α.39. Other characteristics of the species of the genus *Ehrlichia*.

	<i>E. canis</i>	<i>E. chaffeensis</i>	<i>E. ewingii</i>	<i>E. muris</i>	<i>E. ruminantium</i>
Geographic distribution	Worldwide	North America	North America	Japan	Africa, Caribbean
Host(s) with clinical signs	Dogs, other canids	Humans, dogs	Dogs, humans	Mice	Cattle, sheep, goats, other ruminants
Reservoir host(s)	Canids	Cervids, canids	Dogs	Mice	Ruminants
Vectors	<i>Rhipicephalus sanguineus</i>	<i>Amblyomma americanum</i> , <i>Dermacentor variabilis</i> ?	<i>Amblyomma americanum</i>	<i>Haemaphysalis flava</i>	<i>Amblyomma variegatum</i> , <i>Amblyomma cajennense</i>
Host cell	Monocytes and macrophages	Monocytes and macrophages	Neutrophils	Macrophages	Endothelial cells and neutrophils
<i>In vitro</i> cultivation	Tick cells, macrophages	Tick cells, macrophages, endothelial cells, fibroblasts	Not cultivated <i>in vitro</i>	Macrophages	Endothelial cells, neutrophils, tick cells

function of the polymorphic proteins that vary from 28–32 kDa is not known, but owing to the presence of multiple copies, it is speculated that these proteins might play a role in immune evasion.

Tetracycline antibiotics are effective at eliminating infection *in vitro* but may have some limitations *in vivo*. Other antibiotics, including imidocarb dipropionate, are not effective.

Infection by *E. canis* stimulates the production of antibodies that also strongly react with *E. chaffeensis*, *E. muris*, *E. ruminantium*, and *E. ewingii* and react more weakly with *Anaplasma phagocytophilum* and *Neorickettsia* spp. Since infection results in low-level persistence, natural immunity is often incomplete, and recrudescence can occur months to years after primary infection. Immunity resulting in elimination of a persistent infection may occasionally occur. Different strains of *E. canis* may not induce homologous or heterologous cross-protection. Protection against other *Ehrlichia* species, including *E. chaffeensis*, is not induced by infection with *E. canis*.

E. canis is the causative agent of canine monocytic ehrlichiosis, a severe febrile infection of canids that occurs after tick bites and is associated with pancytopenia. The disease is worldwide in distribution. Asymptomatic human infection has been documented in one case.

Clinical signs of infection occur in canids—including dogs, coyotes, foxes, and wolves, among others. The organisms have not been demonstrated to be infectious for rodents or other small mammals. Persistent infection leading to a carrier “reservoir” state may develop in canids. The major worldwide vector for *E. canis* is *Rhipicephalus sanguineus*, the brown dog tick. Transstadial but not transovarial transmission occurs; thus, the predominant mechanism for natural maintenance involves a mammalian reservoir that is expanded by tick vectors.

Isolation can be achieved by inoculation of leukocytes from infected dogs onto monolayers of canine DH82 histiocyte cells. Infected cells may be stored for months or years frozen at -80°C or for years in liquid nitrogen if maintained in a medium with at least 30% serum and 10% dimethyl sulfoxide. Isolated bacteria may be identified by reaction with polyclonal or monoclonal antibodies or by polymerase chain reaction amplification of conserved *E. canis* genes.

The mol% G + C of the DNA is: 35.3 (by gene sampling).

Type strain: Oklahoma.

GenBank accession number (16S rRNA): M73221.

2. ***Ehrlichia chaffeensis*** Anderson, Dawson, Jones and Wilson 1992a, 327^{VP} (Effective publication: Anderson, Dawson, Jones and Wilson 1991, 2841.)
chaff'fe.en.sis. N.L. fem. adj. *chaffeensis* of Chaffee; pertaining to Fort Chaffee in western Arkansas, where the patient from whom the first isolate was prepared was identified; L. adj. *-ensis* derived from; M.L. adj. *chaffeensis* from Chaffee.

The characteristics are as given for the genus and as listed in Tables BXII.α.38 and BXII.α.39. See also Fig. BXII.α.43. *E. chaffeensis* forms a phylogenetically distinct clade in the genus *Ehrlichia*; it differs from other species by 1.0–1.9% in 16S rRNA gene sequence and by 6.4–17.7% in the *groESL* operon sequence. Possesses polymorphic major surface proteins of approximately 28 kDa. These proteins

are encoded by 21 distinct genes in a multigene family. These genes are similar to those in other ehrlichiae and are more distantly related to those of *Anaplasma* and *Wolbachia* spp. The nucleic acid sequences of the genes encoding the proteins vary between strains. The function of these polymorphic proteins is not known, but it is speculated that they play a role in immune system evasion. The organisms also possess genes that encode major membrane glycoproteins ranging from 120–140 kDa.

Tetracycline and rifamycin are effective at eliminating infection *in vitro*. Aminoglycosides, fluoroquinolones, macrolides, sulfa-containing, and β -lactam antibiotics are not effective *in vitro*.

E. chaffeensis induces strong cross-reactive serologic responses to *E. canis*, *E. ewingii*, *E. muris*, and *E. ruminantium*, and weaker responses to *Anaplasma phagocytophilum*, and possibly to *Rickettsia rickettsii* and *R. typhi*. Serologic responses can be differentiated by protein immunoblotting.

Infection in deer, goats, and dogs may be persistent, and these animals may be important natural reservoirs. Infection in humans is generally self-limited, and persistence is exceedingly rare. Second infections and recrudescence have not been well investigated in animals, but are probably rare in humans.

E. chaffeensis is a causative agent of monocytic ehrlichiosis in humans and dogs. Infection can result in a severe illness with fever, pancytopenia, and occasionally other manifestations after tick bites. The disease appears to be limited to North America although serologic evidence of infection has been reported in humans in Europe and Africa. Asymptomatic and symptomatic canine infections and asymptomatic human infection are well documented. Persistent infection leading to a carrier “reservoir” state may develop in canids and cervids. The major vector for *E. chaffeensis* is *Amblyomma americanum*, the Lone Star tick, although limited data suggests that *Dermacentor variabilis*, the American dog tick, may also play a role in transmission. Transstadial transmission occurs, but transovarial passage has not been documented; thus, the predominant mechanism for natural maintenance involves a mammalian reservoir that is expanded by tick vectors.

Isolation can be achieved by inoculation of infected leukocytes onto monolayers of canine DH82 histiocyte cells. Infected cells can be stored for months or years frozen at -80°C or in liquid nitrogen for years if maintained in medium with at least 30% serum and 10% dimethyl sulfoxide. Isolated bacteria can be identified by reaction with polyclonal or monoclonal antibodies or by polymerase chain reaction amplification of conserved *E. chaffeensis* genes.

The mol% G + C of the DNA is: 33.9 (by gene sampling).

Type strain: Arkansas, ATCC CRL-10679.

GenBank accession number (16S rRNA): M73222.

3. ***Ehrlichia ewingii*** Anderson, Greene, Jones, and Dawson 1992b, 301^{VP} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159.
ew.in'gi.i. N.L. gen. n. *ewingii* of Ewing, named in honor of Sidney A. Ewing for his pioneering work with this agent.

The characteristics are as given for the genus and as listed in Tables BXII.α.38 and BXII.α.39. *E. ewingii* occupies a distinct clade in the genus *Ehrlichia*, with at least 99.7% 16S rRNA gene sequence similarity among strains. *E. ewingii*

differs from other species by 1.0–1.4% in the 16S rRNA gene sequence and by 9.9–15.1% in the *groESL* operon sequence. *E. ewingii* also contains at least one copy of a homolog of the p28 gene family of *E. chaffeensis*.

E. ewingii induces antibodies that strongly cross-react with *E. canis* and *E. chaffeensis* by indirect fluorescent antibody tests but can be differentiated from those species by protein immunoblot analysis.

E. ewingii is a causative agent of granulocytic ehrlichiosis in humans and dogs; infections can result in a mild to moderate illness with fever, pancytopenia, and occasionally other manifestations after tick bites. Dogs develop polyarthritis more often than with *E. canis* infection. The disease has been described only in North America. *E. ewingii* may be transmitted by *Amblyomma americanum* (Lone Star tick) under experimental conditions.

No *in vitro* isolation has been accomplished, although limited *in vitro* replication of *ex vivo*-infected neutrophils has been described. The organism may be maintained by intravenous inoculation of dog blood that contains infected neutrophils or from “carrier” dogs.

The mol% G + C of the DNA is: 40.6 (by gene sampling).

Type strain: Stillwater.

GenBank accession number (16S rRNA): M73227.

4. **Ehrlichia muris** Wen, Rikihisa, Mott, Fuerst, Kawahara and Suto 1995b, 254^{VP} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159. *mu'ris*. L. gen. n. *muris* of the mouse; the species was first isolated from a mouse.

The characteristics are as given for the genus and as listed in Tables BXII.α.38 and BXII.α.39. *E. muris* occupies a distinct clade in the genus *Ehrlichia*, with at least 99.7% 16S rRNA gene sequence similarity among different strains. *E. muris* differs from other species by 1.0–1.9% in the 16S rRNA gene sequence and by 6.4–15.3% in the *groESL* operon sequence. The organisms possess polymorphic major surface proteins of approximately 28 kDa that are encoded by at least two distinct genes; these genes are similar to those in other *Ehrlichia* and more distantly related to those of *Anaplasma* and *Wolbachia* spp.

Tetracyclines effectively prevent infection of mice, but penicillin, aminoglycosides, and sulfonamides do not.

E. muris induces antibodies that cross-react strongly with *E. canis* and *E. chaffeensis* by indirect fluorescent antibody tests and weakly with *Neorickettsia sennetsu*. Antibodies stimulated by *E. muris* infection may be differentiated by protein immunoblot analysis from those elicited by other *Ehrlichia* spp.

E. muris is a pathogen that causes mild to severe clinical signs—including splenomegaly and lymphadenopathy—in wild mice of Japan and perhaps other regions in Asia. The organisms can be detected in splenic and peritoneal macrophages of infected mice. *E. muris* can be transmitted by *Haemaphysalis flava* ticks under natural conditions.

E. muris can be cultivated in the canine histiocyte cell line DH82 maintained in minimal essential medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine; cultures are incubated at 37°C under an air atmosphere enriched with 5% CO₂. No growth occurs in chicken embryo yolk sacs.

The mol% G + C of the DNA is: 38.5 (by gene sampling).

Type strain: AS145, ATCC VR-1411.

GenBank accession number (16S rRNA): U15527.

5. **Ehrlichia ruminantium** (Cowdry 1925) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2158^{VP} (*Rickettsia ruminantium* Cowdry 1925, 231; *Cowdria ruminantium* Moshkovski 1947, 62.) *ru.mi.nan'ti.um*. M.L. gen. pl. n. *ruminantium* of *Ruminantia*, formerly a common name for cud-chewing animals.

The characteristics are as given for the genus and as listed in Tables BXII.α.38 and BXII.α.39. *E. ruminantium* forms a distinct clade in the genus *Ehrlichia*, sharing at least 99.1% nucleotide sequence similarity in the 16S rRNA gene among strains. *E. ruminantium* differs from other species by 1.3–1.6% in the 16S rRNA gene sequence and by 14.5–17.7% in the *groESL* operon sequence. The organisms possess polymorphic major surface proteins encoded by multigene families similar to those in other *Ehrlichia* species and more distantly related to those in *Anaplasma* and *Wolbachia* spp. The function of the polymorphic proteins, which average approximately 32 kDa, is not known, but it is speculated that they might play a role in immune evasion.

Tetracycline antibiotics are effective at eliminating infection *in vivo*.

Infection by *E. ruminantium* stimulates the production of antibodies that also strongly react with *E. chaffeensis* and *E. canis* but react more weakly with *Anaplasma phagocytophilum*, *A. marginale*, *Rickettsia* spp., *Coxiella burnetii*, and *Neorickettsia* spp. Since infection results in low-level persistence, natural immunity is often incomplete and may result in recrudescence months or years after primary infection. Different strains of *E. ruminantium* may not induce homologous or heterologous cross-protection potentially owing to antigenic diversity in this species.

E. ruminantium is the causative agent of heartwater or cowdriosis, a severe febrile infection of ruminants that occurs after tick bites. The disease is associated with fever, organ dysfunction, and severe neurological signs. The disease is limited to sub-Saharan Africa and the Caribbean. Infection of exotic and domestic ruminants is generally severe, although some native ruminant species are refractory to clinical signs or develop only mild illness. Some strains are pathogenic for mice. Persistent infection leading to a carrier “reservoir” state often develops.

The major vectors for *E. ruminantium* are *Amblyomma* ticks, in particular *A. variegatum* and *A. habreum*. Transstadial but not transovarial transmission occurs; thus, the predominant mechanism for natural maintenance involves horizontal transmission through vertebrates.

Isolation can be achieved by inoculation of blood from infected animals onto monolayers of cultured endothelial cells. Infected cells can be stored for months or years when frozen at –80°C or for years in liquid nitrogen. Isolated bacteria can be identified by reaction with antibodies or by polymerase chain reaction amplification of conserved *E. ruminantium* genes.

The mol% G + C of the DNA is: 32.2 (by gene sampling).

Type strain: Welgevonden.

GenBank accession number (16S rRNA): X61659 (Crystal Springs strain).

Genus III. *Neorickettsia* Philip, Hadlow and Hughes 1953, 257^{AL} emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157

YASUKO RIKIHISA, J. STEPHEN DUMLER AND GREGORY A. DASCH

Ne.o.rick.ett' si.a. Gr. pref. *neo-* new; M.L. fem. n. *Rickettsia* type genus of the family, *Rickettsiaceae*; M.L. fem. n. *Neorickettsia* the new *Rickettsia*

Coccoid or pleomorphic cells that reside in cytoplasmic vacuoles within monocytes and macrophages of dogs, horses, bats, and humans. Small (0.2–0.4 μ m) electron dense forms and relatively large (0.8–1.5 μ m) lighter forms may occur. Tissues of adult **trematode (fluke) vectors** and all other fluke stages—eggs, miracidia, rediae, sporocysts, cercariae, and metacercariae—produce infection when injected into susceptible hosts, indicating transovarial transmission in the vector. Transstadial transmission also occurs. Gram negative. Nonmotile. Most species are cultivable in peripheral blood monocytes, myelomonocytic cell lines, or promyelocytic cell lines. Some species are pathogenic to laboratory mice. **Etiologic agents of diseases of dogs and other canids, horses, and humans.** Where studied, these bacteria possess an antigenically cross-reactive group-specific protein gene with a high degree of sequence identity. Sensitive to tetracycline antibiotics. The estimated genome size is 860–900 kb.

The mol% G + C of the DNA is: 42.

Type species: *Neorickettsia helminthoeca* Philip, Hadlow and Hughes 1953, 257.

FURTHER DESCRIPTIVE INFORMATION

Phylogeny By 16S rRNA gene and *groESL* sequence analysis, the genus *Neorickettsia* forms a clade distinct from *Anaplasma*, *Ehrlichia*, *Wolbachia*, *Rickettsia*, and *Orientia* in the *Alphaproteobacteria*. When 16S rRNA gene sequences are aligned, all species in the genus are more than 95% similar. The amino acid sequence identity between GroEL of *N. sennetsu* and *N. risticii* is 97.6%; however, the identity of this gene between *Neorickettsia* spp. and *Ehrlichia* spp. or *Anaplasma* spp. is no greater than 59.2%.

Cell morphology *Neorickettsia* species are found in the cytoplasm of monocytes in the blood and in macrophages of lymphoid tissues of infected *Canidae*, humans, bats, and horses. In Romanowsky-stained preparations the organisms are dark blue to purple. Individual round forms are most common, but small clusters (microcolonies) of bacteria in inclusions called morulae are also present. These may be mistaken for large bacteria. The morulae are less compact and are smaller than those of *Ehrlichia* spp. in DH82 cells (Rikihisa et al., 1991).

Ultrastructure By electron microscopy small (0.2–0.4 μ m) dense forms and relatively large (0.8–1.5 μ m) light forms have been recognized in several species of *Neorickettsia* (Rikihisa et al., 1985, 1991; Rikihisa, 1990b). Ribosomes, fine DNA strands, and two layers (outer and inner) of membrane, are present in the organisms (Rikihisa, et al., 1991) (see Fig. BXII.α.44). *Neorickettsiae*, like the members of the genera *Ehrlichia* and *Anaplasma*, show no thickening of the inner leaflet of the outer membrane or the outer leaflet of the inner membrane (Rikihisa, 1990b; Rikihisa et al., 1985, 1991). *Neorickettsia* species multiply by binary fission and are found in early endosomes enriched with transferrin receptors in the cytoplasm (Barnewall et al., 1999). Individual organisms or groups of organisms are tightly enveloped by the endosomal membrane. Genes encoding lipid A biosynthesis were not detected, and most genes required for biosynthesis of peptidoglycan were absent (Lin and Rikihisa, 2003).

Cultural characteristics *Neorickettsia* species have not been grown in ordinary bacteriological media or in yolk sacs. Where tested most species are cultivable in peripheral blood monocytes, peritoneal macrophages, myelomonocytic cell lines, or promyelocytic cell lines such as P338D, DH82, and U937 (Rikihisa, 1991b).

Metabolism Members of the genus *Neorickettsia* possess an aerobic type of metabolism and are asaccharolytic. The metabolic activities of *N. risticii* and *N. sennetsu* have been investigated. *Neorickettsiae* can utilize glutamine and glutamate and can generate ATP (Weiss et al., 1988, 1989) as rickettsiae do, but unlike rickettsiae, *neorickettsiae* prefer to use glutamine rather than glutamate. They exhibit this preference because the organisms are enveloped by the host membrane and because glutamine enters endosomes more readily than glutamate. Like the genus *Rickettsia*, the genus *Neorickettsia* cannot utilize glucose-6-phosphate or glucose. The greatest metabolic activity of the *Neorickettsia* is observed at pH 7.2 to 8.0 and declines rapidly below pH 7 (Weiss et al., 1988); thus the genus *Neorickettsia* is not acidophilic.

Genetics Genome sizes of *N. sennetsu* and *N. risticii* are approximately 880 and 870 kb, respectively (Rydina et al., 1996). The sequences of the 16S rRNA genes of *N. helminthoeca*, *N. sennetsu*, and *N. risticii* have been determined. *GroESL* has been cloned from *N. sennetsu* (Zhang et al., 1997), *N. risticii* (Sumner et al., 1997), and from SF agent and *N. helminthoeca* (Rikihisa, et al., unpublished data). The unique feature of the *groESL* operon of *Neorickettsia* spp. is that there is no spacer between *groES* and *groEL* (*groES* and *groEL* overlap by one base). The HSP70 gene of *N. sennetsu* has been cloned (Zhang et al., 1998).

The genes encoding the 51-kDa protein antigen have been identified in various strains of *N. risticii* from horses and trematodes (Barlough et al., 1998; Dutta et al., 1998; Reubel et al., 1998; Kanter et al., 2000) and in other *Neorickettsia* spp. (Rikihisa et al., unpublished data). Amino acid sequence identities of the 51-kDa protein among *N. risticii* strains are greater than 77% (Kanter et al., 2000). Another antigenic protein, 50/85 kDa in size, shows strain-dependent polymorphism, being 50 kDa in strain 25-D and 85 kDa in strain 90-12 by SDS-PAGE, the higher value is due to the presence of tandem repeat nucleotide sequences which vary in size, number and type of repeats (Biswas et al., 1998). Genes encoding a type IV secretion apparatus and two-component regulatory systems were identified (Rikihisa, unpublished data).

Antigenic structure Indirect fluorescent antibody (IFA) and Western immunoblot analyses of purified cells of *N. helminthoeca*, *N. sennetsu* and *N. risticii* using antisera from infected animals have shown that the surface antigens of these organisms are highly cross-reactive (Rikihisa et al., 1991). The cross-reactive antigens between *N. risticii* and *N. sennetsu*, as detected by Western immunoblot analysis, are proteins 51–55 kDa in size (Rikihisa, 1996). The 55-kDa GroEL homolog, the 50/85-kDa strain-specific antigen, and the 51-kDa antigen are in this range (Vemulapalli et al., 1998). The 51- and 50/85-kDa proteins are apparently unrelated. The 51-kDa protein has a putative signal peptide se-

quence and is thus a potential outer membrane protein (Vemulapalli et al., 1998), whereas the 50/85-kDa protein does not. GroEL is also a dominant antigen in *Neorickettsia* spp. and has been shown to cross-react with the GroEL of *Ehrlichia* spp., *Orientia* spp. and *Rickettsia* spp. but not with the GroEL of *Escherichia coli* (Zhang et al., 1997).

Pathogenicity *N. helminthoeca* is the causative agent of salmon poisoning disease of the *Canidae*, *N. sennetsu* is the agent of human Sennetsu ehrlichiosis, and *N. risticii* is the causative agent of Potomac horse fever or equine monocytic ehrlichiosis. When examined with an *in vitro* cultivation assay system, professional phagocytes—monocytes and macrophages—of host animal species have no intrinsic resistance to infection with *Neorickettsia* spp. *Neorickettsia* spp. also cause systemic febrile illness with thrombocytopenia and leukopenia, with or without lymphadenopathy. *Neorickettsia* spp. induce intestinal manifestations, which are especially severe with *N. risticii*, which causes watery diarrhea. Evidence of a severe inflammatory reaction is usually absent at necropsy. Pathogenicity has been studied most extensively for *N. risticii*. *N. risticii* specifically binds to macrophages, and both the host cell receptor and the *N. risticii* ligand appear to be proteins (Messick and Rikihisa, 1993). *Neorickettsia* enter macrophages by receptor-mediated endocytosis, not by phagocytosis, and reside in the early endosomes that do not fuse with lysosomes (Wells and Rikihisa, 1988; Messick and Rikihisa, 1993; Rikihisa et al., 1994). *Neorickettsia* internalization and proliferation are dependent on Ca^{2+} -calmodulin-signaling and protein tyrosine phosphorylation (Rikihisa et al., 1995; Zhang and Rikihisa, 1997). *N. sennetsu* resides in early endosomes enriched with transferrin receptor and upregulates transferrin receptor mRNA by activating iron-responsive protein 1 (Barnewall et al., 1999). This activation might be a mechanism to facilitate iron uptake by *neorickettsiae*.

Neorickettsia release appears to occur not only by cell lysis but also through exocytosis by fusion of the inclusion membrane with the plasma membrane. In the case of intestinal epithelial cells, which make a monolayer tightly connected by circumferential zones of intercellular junctions, *neorickettsiae* appear to be transmitted between adjacent cells *in vitro* by a coupled exocytosis in one cell and endocytosis in an adjacent cell (Rikihisa, 1990a). *N. risticii* can be consistently reisolated from equine peripheral blood monocytes from days 1–28 after infection and 8 days after spontaneous resolution of clinical signs (Mott et al., 1997). *N. risticii* may persist much longer, since in a pregnant mare-fetus system, *N. risticii* has been isolated from the aborted fetus of a mare that was experimentally infected up to 3.5 months prior to the abortion (Dawson et al., 1987; Long et al., 1995).

Mouse pathogenicity of *Neorickettsia* spp. varies. *N. sennetsu* is pathogenic for mice. Clinical signs are ruffled fur, inactivity, and mild diarrhea followed by death in about 3–4 weeks after inoculation (Misao and Kobayashi, 1954). Necropsy of mice reveals marked enlargement of the spleen, the liver, and lymph nodes throughout the body. All human isolates have been obtained from the peritoneal macrophages of the mouse after intraperitoneal inoculation of patient's blood. *N. helminthoeca* is not pathogenic for mice. *N. risticii* can establish infection in mice and horses with or without causing apparent clinical illness. Based on a murine model for Potomac horse fever, the disease is apparently dose-dependent, i.e., with a low dose of *N. risticii*, innate defense mechanisms appear to contain *Neorickettsia* cells below a threshold level. Only at higher doses can the organism cause

disease and pathological changes (Rikihisa et al., 1987). Horses that recover are immune to Potomac horse fever for up to 20 months after infection (Palmer et al., 1990). Exposure to killed organisms can protect mice from *N. risticii* infection (Rikihisa, 1991a). Both humoral and cell-mediated immune responses appear to play significant roles in this protection. The presence of neutralizing antibodies and antibody-dependent cellular toxicity has been demonstrated using a cell culture system (Messick and Rikihisa, 1992a; Rikihisa et al., 1993, 1994) and a murine model (Rikihisa et al., 1993). Polyclonal antiserum to *N. risticii* does not inhibit binding of *N. risticii* cells to P388D macrophages or their internalization, but internalized antibody-coated *N. risticii* fails to survive (Messick and Rikihisa, 1994). Because macrophages bear the Fc receptor, the Fab fragment of the antibody was used to analyze the inhibitory mechanism. Since the Fab fragment of the anti-*N. risticii* IgG blocks the binding of *N. risticii* to macrophages, antibody-coated *N. risticii* most likely enters macrophages via the Fc receptor. Fc-receptor-mediated uptake may deliver *N. risticii* to the intracellular compartment that is susceptible to lysosomal fusion. Alternatively, because ^{14}C -L-glutamine metabolism by purified host cell-free *N. risticii* is blocked by polyclonal antibody, direct metabolic inhibition may make *N. risticii* unable to survive in the macrophage (Messick and Rikihisa, 1994). *N. risticii* is very sensitive to nitric oxide, which is generated by macrophage cytoplasmic nitric oxide synthase induced by interferon gamma ($\text{IFN-}\gamma$) (Park and Rikihisa, 1991, 1992). Activities of T cells that generate $\text{IFN-}\gamma$ are, however, generally severely depressed in infected mice in a time- and dose-related manner (Rikihisa et al., 1987). One cause for depressed T-cell response was found in macrophages. Class II histocompatibility antigen (Ia antigen) induction on the surface of *N. risticii*-infected macrophages (antigen-presenting cells) is suppressed *in vitro* (Messick and Rikihisa, 1992b), suggesting inhibition of antigen-specific T-cell activation in *N. risticii* infection. *N. risticii* induces only low levels of tissue necrosis factor alpha ($\text{TNF-}\alpha$) and prostaglandin E2 production in murine macrophages *in vitro* (van Heeckeren et al., 1993). In contrast, infected macrophages produce significant amounts of interleukin 1 (IL-1).

Ecology Where known, *Neorickettsia* spp. are transmitted and maintained in trematodes found in *Pleuroceridae* snails (Nyberg et al., 1967; Millemann and Knapp, 1970). Infection of mammals is required for completion of the life cycle of the trematodes, but it is not directly required for maintenance of *Neorickettsia* spp. Dogs, horses, bats, and humans accidentally acquire *Neorickettsia* spp. by ingestion of the metacercaria stage of trematodes in fish or adult aquatic insects. The life cycle of *N. helminthoeca* includes growth in eggs of a digenetic microcercous trematode, *Nanophyetus salmincola*, in the redia stage of the trematode in the snail *Oxytrema silicula*, in the metacercaria stage of the trematode in salmonid fish, and in monocytes and macrophages in the dog. *N. helminthoeca* infects neither the fish nor the snail carrying the trematode. The disease has been reported from the Pacific Coast of Northern California, Oregon, and Washington. Additionally, several salmon poisoning cases have been reported in Vancouver Island, Canada (Booth et al., 1984).

N. risticii has been found in digenetic and virgulate trematodes of the family *Lecithodendoriidae*, which infect snails in the *Pleuroceridae* family, namely, *Elimia* and *Juga* spp., in the midwestern and northeastern U.S. and in California, respectively (Barlough et al., 1998; Reubel et al., 1998; Kanter et al., 2000). *N. risticii*-infected trematodes then penetrate and develop into metacer-

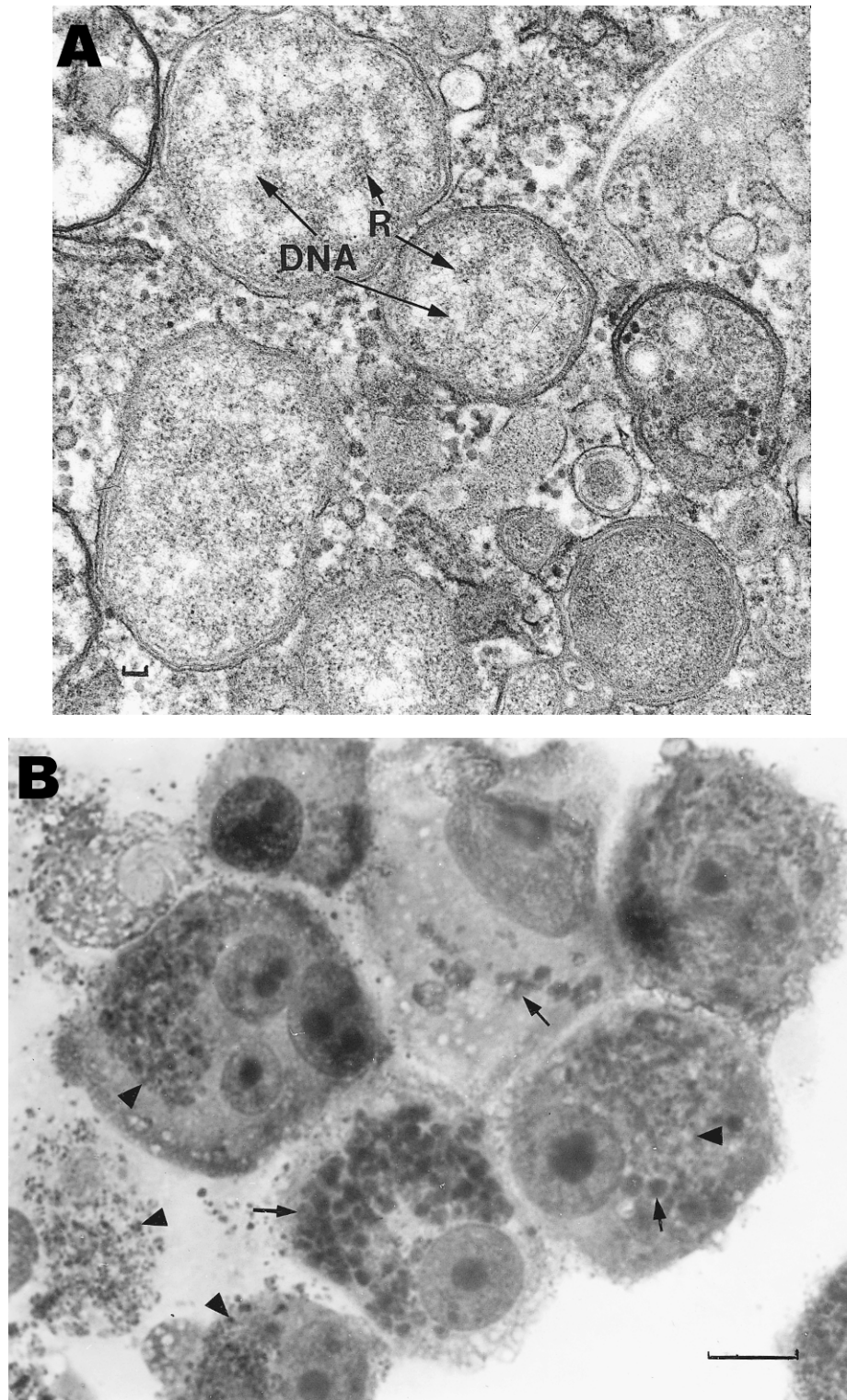


FIGURE BXII.α.44. *Neorickettsia helminthoeca*. A, Ultrathin section of an infected, cultured, canine monocytic cell (cell line DH82). *N. helminthoeca* organisms are tightly enveloped by a host cell membrane (*arrowheads*). Each bacterium is surrounded by a distinct plasma membrane and an outer membrane. Fine DNA strands and ribosomes (*R*) are evident in the organisms. ($\times 37,000$). B, *N. helminthoeca* are seen in the cytoplasm of DH82 cells as small inclusions containing several organisms (*arrows*), as individual organisms (*arrowheads*), and as extracellular organisms. Preparation stained by Diff-Quik stain ($\times 1,275$) (Reproduced with permission from Y. Rikihisa, et al., *Journal of Clinical Microbiology* 29: 1928–1933, 1991, American Society for Microbiology, Washington, D.C.)

cariae in nymphs of a variety of aquatic insect including mayflies, caddis flies, and stone-flies (Chae et al., 2000). *N. risticii* survives through metamorphosis of insects and persists in adult insects; horses can acquire Potomac horse fever by ingestion of infected adult caddis flies (Madigan et al., 2000a; Mott et al., 2002). Despite epidemiological evidence that patients acquire Sennetsu fever by eating raw gray mullet fish, *N. sennetsu* has not been isolated from trematodes. However, the *Stellantochasmus falcatus* (SF) agent, which is more closely related to *N. risticii* than to *N. sennetsu*, has been isolated directly from metacercariae of the *S. falcatus* trematode in the gray mullet fish in a Sennetsu fever-endemic region in Japan (Fukuda et al., 1973; Yamamoto, 1978; Wen et al., 1996).

Antibiotic sensitivity All *Neorickettsia* spp. are sensitive to tetracycline antibiotics; however, they are not susceptible to β -lactam antibiotics, aminoglycosides, macrolides, chloramphenicol, or sulfonamides (Rikihisa and Jiang, 1988, 1989).

ENRICHMENT AND ISOLATION PROCEDURES

N. helminthoeca has been isolated from the blood, spleen, and liver of dogs that have been infected by feeding them metacercariae-infected salmon; the organism was then propagated in the canine myelocytic leukemia cell line DH82 (Rikihisa et al., 1991). *N. risticii* has been isolated from the blood of horses infected with canine peripheral blood monocytes, U937 cells, or P388D cells (Rikihisa and Perry, 1984, 1985; Dutta et al., 1985; Holland et al., 1985). *N. risticii* has also been isolated from the blood of horses infected by oral administration of adult caddis flies or by subcutaneous inoculation of cercaria or sporocysts obtained from snails collected in an endemic area (Madigan et al., 2000a; Pusterla, et al., 2000b). *N. sennetsu* was isolated by intraperitoneal inoculation of patients' blood into laboratory mice (Fukuda et al., 1954; Misao and Kobayashi, 1954). The SF agent has been directly isolated by oral or intraperitoneal inoculation of mice with metacercariae excised from gray mullet fish (Fukuda et al., 1973).

MAINTENANCE PROCEDURES

The organism can be preserved by storing infected primary blood monocyte cultures or infected DH82 or P388D cell cultures in liquid nitrogen. The organism can also be preserved by storage at -80°C of a 20% suspension of homogenized infected lymph node or spleen tissue in RPMI 1640 medium containing 10% dimethyl sulfoxide and 15% fetal bovine serum (Rikihisa et al., 1991).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *NEORICKETTSIA*

Diagnostic features of *Neorickettsia* spp. are shown in Table BXII.α.40. The preferred methods for definitive identification of *Neorickettsia* species are sequence analysis of all or part of 16S rRNA gene, the *groESL* operon, or the *gltA* gene, or alternatively,

DIFFERENTIATION OF THE GENUS *NEORICKETTSIA* FROM OTHER GENERA

Neorickettsiae are distinguished from *Ehrlichia*, *Anaplasma*, and *Wolbachia* spp. by their 16S rRNA gene sequences or *groESL* gene sequences, by the presence of a 51-kDa protein gene unique to *N. risticii*, *N. sennetsu*, and SF agent, by the lack of *msp2* or p44 homologs of *Anaplasma* spp. (Zhi et al., 1999) or OMP-1 homologs of *Ehrlichia* spp. (Ohashi et al., 1998a), by not being tickborne, and by their life cycle in trematodes.

TAXONOMIC COMMENTS

Sequence comparisons of almost complete 16S rRNA genes revealed that the sequence similarity of a horse isolate of *N. risticii*—isolate 081—to *N. sennetsu* Miyayama is 99.7%—slightly greater than the similarity to either the *N. risticii* type strain (99.6 %) or to *N. risticii* Kentucky. The 16S rRNA gene sequence similarities between *N. helminthoeca* and *N. sennetsu* strains or *N. risticii* strains are 95.4–95.8% (Wen et al., 1996). According to Stackebrandt and Goebel (1994), a 16S rRNA gene sequence similarity of less than 97% between strains indicates that they represent different species, but at 97% or higher 16S rRNA gene sequence similarity, DNA relatedness must be used to determine whether strains belong to different species. Therefore, *N. sennetsu* and *N. risticii* belong to the same species based on 16S rRNA gene sequences.

The 16S rRNA gene sequence similarity between *N. risticii*, the agent of Rocky Mountain spotted fever, and *Neorickettsia* spp. is low (80%) (Pretzman et al., 1995).

FURTHER READING

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amplification of portions of these genes by the polymerase chain reaction using species-specific oligonucleotide primers. Species-specific primers for *N. helminthoeca* and *N. risticii* and *N. sennetsu* are shown in Table BXII.α.41.

List of species of the genus *Neorickettsia*

1. *Neorickettsia helminthoeca* Philip, Hadlow and Hughes 1953, 257^{AL}

hel.minth'oe.ca. Gr. n. *helmins*, *helminthis* worm; Gr. n. *oikos* house; M.L. fem. adj. *helminthoeca* worm-dwelling.

TABLE BXII.α.40. Descriptive and diagnostic features of *Neorickettsia* species^a

Characteristic	<i>N. helminthoeca</i>	<i>N. risticii</i>	<i>N. sennetsu</i>
Geographic distribution	U.S.A., Canada	North and South America, India, Europe	Japan, Malaysia
Reservoir host(s) and vectors	<i>Nanophyetus salmincola</i>	<i>Lecithodendriidae</i> trematodes	unknown trematode
Usual host species and infected host(s) that show clinical signs	Canidae	Horses	Humans
Usual infected host cell type	Monocytes	Monocytes, intestinal epithelial cells, mast cells	Monocytes
Presence in peripheral blood	+	+	+
Serological reaction with:			
<i>N. helminthoeca</i> antiserum	+++	++	++
<i>N. risticii</i> antiserum	++	+++	+++
<i>N. sennetsu</i> antiserum	++	+++	+++

^aSymbols: +, low; ++, medium; +++ high.**TABLE BXII.α.41.** Species-specific oligonucleotide primers for PCR identification of *Neorickettsia* species

Species	Target gene	Forward primer	Reverse primer
<i>N. helminthoeca</i>	16S rRNA ^a	5'-GGACTTTTGGCTGCTTGCCAG-3'	5'-TGGGTACCGTCATTATCTTCC-3'
<i>N. risticii</i>	16S rRNA ^a	5'-GGAATCAGGGCTGCTTGCCAGC-3'	5'-TGGGTACCGTCATTATCTTCC-3'
	<i>groESL</i> ^b	5'-GGTTACAAGGTAATTAACAAC-3'	5'-CGGCAATCTTGTTACCGATT-3'
<i>N. sennetsu</i>	16S rRNA ^a	5'-GGAATCAAAGCTGCTTGCCAG-3'	5'-TGGGTACCGTCATTATCTTCC-3'
	<i>groESL</i> ^b	5'-GGTTATAAGGTGATGAATCAG-3'	5'-CGGCAATCTTGCCACCAATC-3'

^aAnderson et al. (1992c); Pretzman et al. (1995); Kanter et al. (2000).^bSumner et al. (1997); Zhang et al. (1997).

The characteristics are as described for the genus and as listed in Table BXII.α.40. *N. helminthoeca* is the most phylogenetically distinct species in the genus *Neorickettsia*, differing from other species by approximately 4.5% in the 16S rRNA gene sequence. *N. helminthoeca* causes salmon poisoning disease, an acute and highly fatal disease of domestic and wild canidae. Clinical signs are fever, anorexia, inactivity, depression, vomiting, and diarrhea. In infected dogs, the major lymph nodes are enlarged. Although the trematode vector infects a wide variety of species of animals, salmon poisoning disease occurs chiefly in *Canidae* (dogs, coyotes, foxes) and is occasionally seen in immunosuppressed individuals of other animal species. Venereal transmission from an infected male to a female has also been reported. Rectal and aerosol transmission is also possible. Blood, feces, and lymph node aspirates are infectious; thus infected dogs must be isolated and care used so as not to induce iatrogenic transmission.

Ingested metacercariae in salmonid fish mature in 5–6 days in the gut, and the adult stage attaches deep in the interior of intestinal mucosa, inducing inflammation, i.e., hyperemia, inflammatory cell migration, and edema around the parasites. By an unknown mechanism, *N. helminthoeca* is transferred to monocytes and macrophages, which migrate through blood and lymphatic vessels and lodge in somatic and visceral lymph nodes. *N. helminthoeca* circulates in the blood of orally infected dogs starting day 8–12 after infection, as evidenced by the successful re-isolation of *N. helminthoeca* from the blood from day 8–11 after infection until death (Rikihisa et al., 1991). Lymph nodes appear to be the primary site of multiplication of *neorickettsiae*. Like *N. risticii*, *N. helminthoeca* appears to exhibit intestinal tissue tropism, since not only the oral route of infection but also the intravenous inoculation of cell-cultured organisms induces severe hemorrhage throughout the small intestine. Nonsuppurative meningitis or meningoencephalitis and brain lesions have also been noted in

both natural and experimental *N. helminthoeca* infections. The severe central nervous system depression seen in salmon poisoning disease is probably related to these brain lesions. Dogs experimentally infected with *N. helminthoeca* P.O. develop IgG antibody 13–15 days after infection as detected by IFA testing (Rikihisa et al., 1991).

Clinical signs are characterized by anorexia, inactivity, depression, and sudden onset of fever above 39.8°C and up to 42.7°C, followed by hypothermia over the next 4–8 days. Vomiting may precede the typical watery yellowish diarrhea that is sometimes tinged with blood. Rapid weight loss is evident and the lack of ingestion of water, coupled with fluid loss through vomiting and diarrhea, results in severe dehydration with both electrolyte and acid-base imbalances. Serous nasal and ocular discharges are sometimes seen. Lymphadenopathy of the major nodes is easily palpable.

Microscopically, lesions are generally mild and proliferative in character, with very little evidence of degeneration and necrosis in the various organs including the brain, although extensive necrosis may accompany the proliferative response in lymphoid tissue. Lesions consist of mononuclear cell infiltration. Generalized lymph node enlargement is due to marked infiltration of macrophages, accompanied by severe depletion of small lymphocytes and a loss of germinal centers.

Elokomin fluke fever agent was reported to occur along the Elokomin River in the state of Washington. The agent has a wider host range than *N. helminthoeca* and is reported to infect bears, raccoons, and ferrets in addition to the *Canidae*. Clinical signs of Elokomin fluke fever are milder than those of salmon poisoning disease. The agent has not been cultured in a continuous cell line. It is currently considered a strain of *N. helminthoeca* but much information is lacking (Rikihisa and Zimmerman, 1995).

The mol % G + C of the DNA is: 42 (by gene sampling).

Type strain: no culture isolated.

GenBank accession number (16S rRNA): U12457.

2. **Neorickettsia risticii** (Holland, Weiss, Burgdorfer, Cole and Kakoma 1985) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159^{VP} (*Ehrlichia risticii* Holland, Weiss, Burgdorfer, Cole and Kakoma 1985, 524.) *ris.ti'cii*. M.L. n. *ristic* from Miodrag Ristic, meaning Ristic's.

The characteristics are as described for the genus and as listed in Table BXII.α.40. *N. risticii* cells are generally round, sometimes pleomorphic, and may be elongated, especially in tissue culture. The cells divide by binary fission. As determined by electron microscopy and immunoperoxidase staining, the organisms are found in membrane-lined vacuoles within the cytoplasm of infected eucaryotic host cells, primarily macrophages, mast cells, and glandular epithelial cells in the intestine of the horse (Rikihisa et al., 1984, 1985; Steele et al., 1986). *N. risticii* cells are shed into the intestinal lumen (Rikihisa et al., 1985) and are found in feces (Mott et al., 1997). Morulae and individually enveloped forms appear to transform into one another. An intermediate stage has been described (Rikihisa et al., 1985); it appears similar to a moderately electron-dense ehrlichia that is tightly enveloped by a host membrane. The membrane is continuous with a membrane surrounding a morula. *N. risticii* cells are primarily seen as individual forms in the T-84, P388D, and U-937 cell lines, in primary equine monocyte culture, and in infected equine tissues. The individual form is especially common in intestinal epithelial cells.

N. risticii is the causative agent of Potomac horse fever, which is also called equine monocytic ehrlichiosis. The disease may also be called equine ehrlichial colitis and acute equine diarrhea syndrome, depending on the clinical signs displayed in a given horse. Serological data indicate that Potomac horse fever occurs in 43 of the United States, in two provinces in Canada (Ontario and Saskatchewan), and in France, Italy, Venezuela, India, Brazil, and Australia (Rikihisa et al., 1990; Ristic, 1990).

Clinical signs are an acute onset of fever (up to 107°F), depression, anorexia, decreased borborygmi in all abdominal quadrants, subcutaneous edema of the legs and ventral abdomen, dehydration, and diarrhea (Rikihisa, 1997). Laminitis and severe abdominal pain occur in 15–25% and 5–10% of cases, respectively. Development of these signs is the most common reason for euthanizing infected horses. Laminitis may progress, even when other clinical signs resolve. Diarrhea may be mild to severe (pipestream), and occurs in 10–30% of cases. In some horses diarrhea is transient; in others, it persists for several days. Some horses may have no diarrhea. The case fatality rate ranges from 5–30%. Transplacental transmission of *N. risticii* occurs and the organism may induce abortion or resorption of the fetus or infect foals, which then require extensive neonatal care. Leukopenia and rebound leukocytosis are prominent hematological changes. Anemia, plasma protein concentration, and packed cell volume may increase. Thrombocytopenia may also be observed. Pathogenicity in laboratory mice varies among strains.

Watery diarrhea is caused by a reduction in electrolyte transport (Na^+ , Cl^-); thus, there is lack of water resorption, mainly in the large and small colons (Rikihisa et al., 1992). Infected intestinal epithelial cells lose microvilli, which may

contribute to the reduced electrolyte transport and water resorption. An increase in intracellular cyclic AMP (cAMP) is found in both infected mouse macrophages and infected horse intestinal tissues. This change in cAMP content may also contribute to the reduced luminal absorption of Na^+ and Cl^- in the colon, and thus to the lack of water absorption and diarrhea.

Genetic and antigenic divergence among strains has been examined (Chaichanasiriwithaya et al., 1994). The Ohio 081 strain was significantly different from Maryland, Virginia, Kentucky, or other Ohio strains in protein composition and 16S rRNA gene sequence. In agreement with these findings, the identity of the P51 amino acid sequences between the type strain and the Ohio 081 strain is 80.6%, the lowest among all P51s examined (Kanter et al., 2000). The greatest 16S rRNA gene sequence difference was between the type strain of *N. risticii* and the Bunn strain, followed by the Ohio 081 strain (14 and 10 bases, respectively) (Wen et al., 1995a). Genetic comparisons of strains do not show segregation of strains among those from the blood of horses, those from snails or trematodes, or those from aquatic insects. Thus, it appears that strains that affect horses are also present in trematodes in snails and insects. The genome size is 880 kb (Rydikina et al., 1996).

The mol% G + C of the DNA is: 42 (by gene sampling).

Type strain: HRC-IL, Illinois, ATCC VR-986.

GenBank accession number (16S rRNA): M21290.

3. **Neorickettsia sennetsu** (Misao and Kobayashi 1956) Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2159^{VP} (*Rickettsia sennetsu* Misao and Kobayashi 1956) *Ehrlichia sennetsu* Ristic and Huxsoll 1984, 355. *sen'ne'tsu*. M.L. n. *sennetsu* from Japanese, meaning glandular fever.

The characteristics are as described for the genus and as listed in Table BXII.α.40. The morphology is similar to that of *N. risticii*. *N. sennetsu* is the causative agent of human sennetsu rickettsiosis fever, also called Hyuga fever and Kagami fever. The clinical aspects may vary from mild headache, slight back pain, and a low-grade fever to a severe form of the disease characterized by persistent high fever, anorexia, lethargy, lymphadenopathy, and prominent hematologic abnormalities. The disease is limited to Western Japan and Malaysia (Ristic, 1990). *N. sennetsu* is not pathogenic for horses, but horses infected with it are protected from developing Potomac horse fever when challenged with *N. risticii* (Rikihisa et al., 1988). The 16S rRNA gene sequences and the *groESL* gene sequences (Zhang et al., 1997) are similar in *N. risticii* and *N. sennetsu*. The mode of transmission of *N. sennetsu* is unknown, although metacercariae that parasitize the gray mullet fish are suspected as the vector because of the association of the disease with the consumption of a raw fish of this type (Fukuda et al., 1973). Mice are highly susceptible to *N. sennetsu* and are used to isolate the organism from human specimens (Fukuda et al., 1954; Misao and Kobayashi, 1954). The genome size is 859 kb based on whole genome sequencing (Rikihisa, unpublished data).

The mol% G + C of the DNA is: 42 (by genome sequencing).

Type strain: Miyayama, ATCC VR-367.

GenBank accession number (16S rRNA): M73219.

Genus IV. *Wolbachia* Hertig 1936, 472^{AL}

BERNARD LA SCOLA, CLAUDIO BANDI AND DIDIER RAOULT

Wolbachia. M.L. fem. n. *Wolbachia* named after S. Burt Wolbach, who described the rickettsial agent of Rocky Mountain spotted fever and, in collaboration with Marshall Hertig, studied the rickettsia-like microorganisms of insects.

Pleomorphic bacteria that appear as small rods (0.5–1.3 µm in length) and coccoid forms (0.25–1 µm); large forms (1–1.8 µm in diameter) may be observed. Grow in vacuoles of host cells. Do not grow outside of cells. Associated with arthropods and filarial nematodes. Responsible for reproductive alterations in arthropods. With few exceptions, not pathogenic for the host. **Strains associated with nematodes responsible for indirect pathogenesis in humans. Susceptibility to doxycycline and rifampin demonstrated *in vitro* and *in vivo*.**

The mol% G + C of the DNA is: not determined.

Type species: *Wolbachia pipientis* Hertig 1936, 472.

FURTHER DESCRIPTIVE INFORMATION

Discovered in 1924 and described in 1936 by Hertig (Hertig and Wolbach, 1924; Hertig, 1936), *Wolbachia pipientis* is currently regarded as the sole species of the genus *Wolbachia* (Dumler et al., 2001). *Wolbachia* is a member of the *Alphaproteobacteria*.

Older descriptions of the genus *Wolbachia* included two species, *Wolbachia persica* and *Wolbachia melophagi*, that have characteristics distinct from *W. pipientis* (Fig. BXII.α.45). *Wolbachia persica* belongs to the *Gammaproteobacteria* (Weisburg et al., 1989; Forsman et al., 1994) and should be reclassified as "*Francisella persica*" (Niebylski et al., 1997a). The exact taxonomic position of *Wolbachia melophagi* has yet to be determined, but its description as an extracellular bacterium in the lumen of its host, *Melophagus ovinus*—a wingless fly commonly called sheep ked (Weiss et al., 1984)—is not in accordance with the strictly intracellular location of bacteria of the family *Anaplasmataceae*. In addition, a 16S rDNA gene sequence obtained from *W. melophagi* shows high homology with the 16S rRNA gene of species in the genus *Bartonella* (R.J. Birtles and D.H. Molyneux, unpublished GenBank accession X89110). A formal proposal to remove *W. persica* and *W. melophagi* from the genus *Wolbachia* has recently been published (Dumler et al., 2001). Descriptions of *Wolbachia persica* and

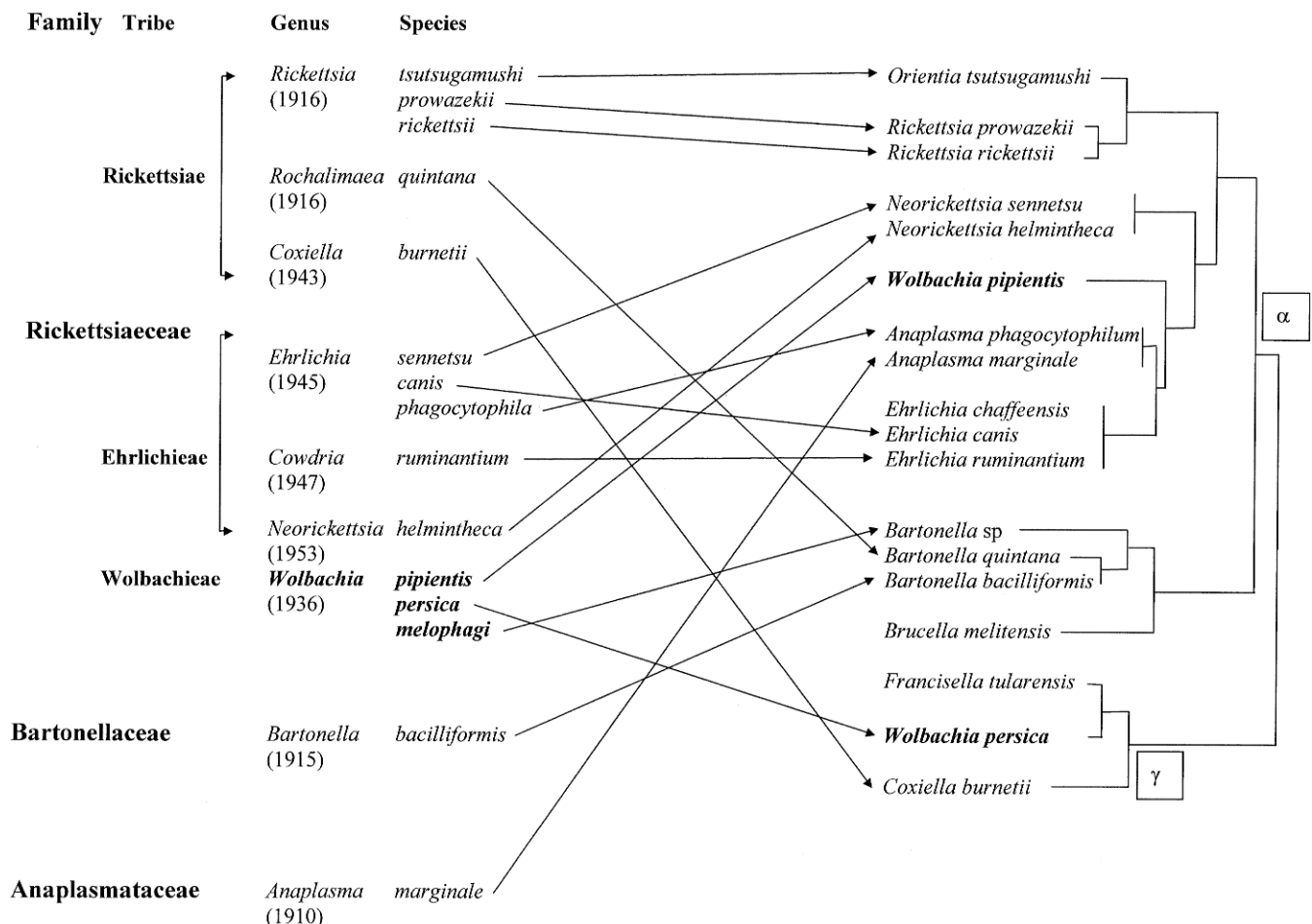


FIGURE BXII.α.45. The left-hand side of the figure indicates the classification of the *Rickettsiae* in *Bergey's Manual*. (Reproduced with permission from E. Weiss et al., *In Krieg and Holt (editors), Bergey's Manual of Systematic Bacteriology*, ©Williams & Wilkins Co., 1984, pp. 711–713). The classification on the right-hand side is based on a comparison of 16S rRNA gene sequences including the recent reorganization in the families *Rickettsiaceae* and *Anaplasmataceae* (Dumler et al., 2001).

Wolbachia melophagi can be found in the chapters on *Francisella* and *Bartonella*, respectively.

Additional *Wolbachia*-like organisms have been described, including “*Wolbachia postica*”, “*Wolbachia trichogrammae*”, and “*Wolbachia popcon*” (Hsiao and Hsiao, 1985; Louis and Nigro, 1989; Min and Benzer, 1997).

W. pipientis is not visible by Gram staining in spite of its Gram-negative cell wall structure (Fig. BXII.α.46). The organisms can be stained using conventional Giemsa’s stain or rapid staining such as Diff-Quick (Fig. BXII.α.47). With the Gimenez stain, the bacteria can be visualized, but they appear as dark blue structures within a blue-green cytoplasm and not as pink-red structures as do rickettsiae. A peptidoglycan layer has not been observed and the cells display some plasticity (Yen and Barr, 1974; Wright et al., 1978; Wright and Barr, 1980). Association with bacteriophage-like particles has been described previously (Wright et al., 1978).

More recently, a bacteriophage-like genetic element, named bacteriophage WO, has been identified in *Wolbachia* (Masui et al., 2000).

W. pipientis multiplies by binary fission in the vacuoles of host cells and is surrounded by a membrane of host origin (Fig. BXII.α.46). In arthropods, the bacteria are present mostly in the cytoplasm of cells in the reproductive organs, but they can also be observed in other tissues, including nervous tissue and hemocytes (Louis and Nigro, 1989; Rigaud et al., 1991). In filarial nematodes, *Wolbachia* is present in the lateral cords and in the female reproductive apparatus (Kozek, 1977; Kozek and Marroquin, 1977). In both arthropods and nematodes, *Wolbachia* is transovarially transmitted to the offspring (Werren, 1997; Bandi et al., 2001).

A strain of *W. pipientis* has recently been established in an *Aedes albopictus*-derived cell line that allows production of signif-

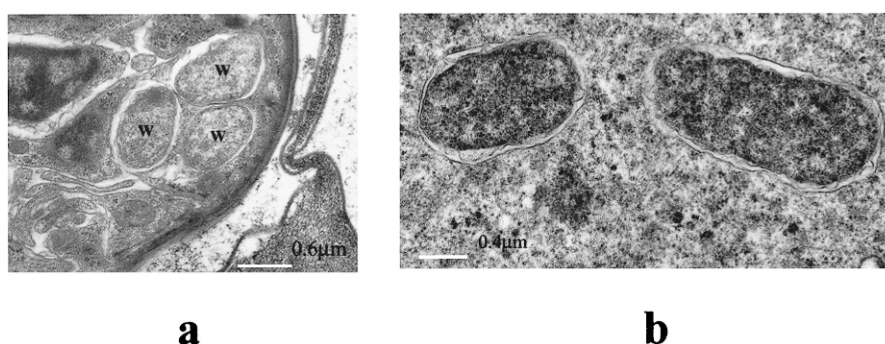


FIGURE BXII.α.46. *Wolbachia pipientis* in filarial nematodes. (a) *Wolbachia* (W) inside an embryo of the dog heartworm *D. immitis*. (b) Details of *Wolbachia* endosymbionts inside an oocyte of the lymphatic filarial worm *Brugia pahangi* (photographs courtesy of Luciano Sacchi).

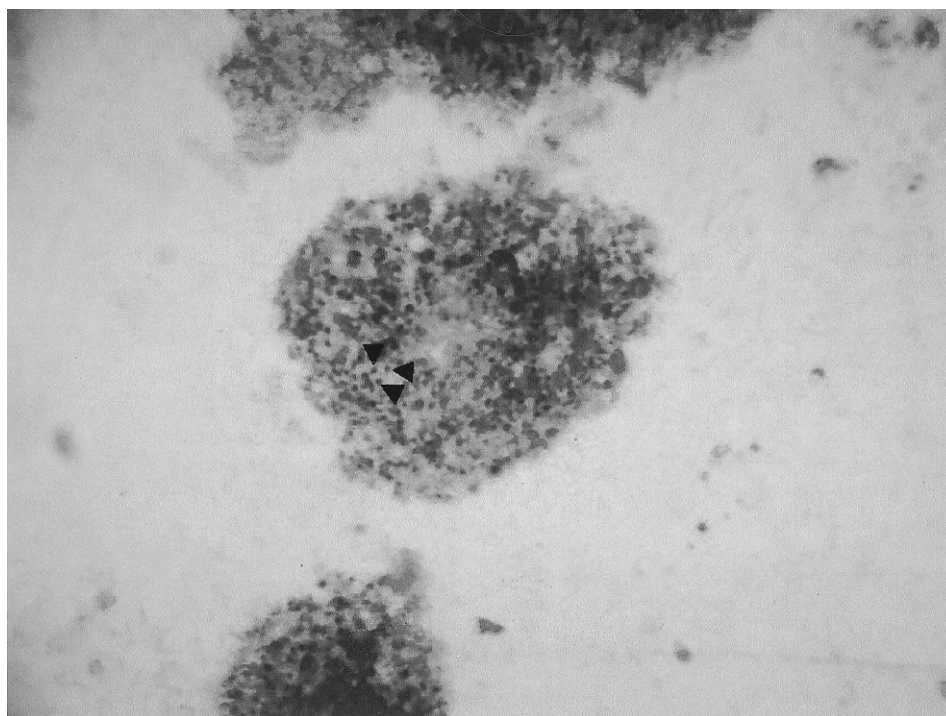


FIGURE BXII.α.47. O'Neill strain of *Wolbachia pipientis* in heavily infected Aa23 cells of *Aedes albopictus* using Giemsa's stain. Three bacterial cells are indicated by arrows.

icant amounts of the bacteria (O'Neill et al., 1997). This strain has been deposited at the American Type Culture Collection (ATCC VR-1529).

W. pipientis was originally observed in the gonads of the mosquito *Culex pipiens* (Hertig and Wolbach, 1924; Hertig, 1936). Later surveys showed *Wolbachia* to be present in at least 16% of sampled insect species (Werren et al., 1995a). The organism has also been found in representatives of other classes of arthropods (Rousset et al., 1992) and more recently in filarial nematodes of the family *Onchocercidae* (Sironi et al., 1995). Several species of filarial nematodes are known to be infected by *Wolbachia* (Table BXII.α.42). *Wolbachia*-infected taxa and the prevalence of infection have been reviewed recently (Taylor and Hoerauf, 1999; Bandi et al., 2001; Stevens et al., 2001) (Table BXII.α.42). In arthropods, *Wolbachia* is patchily distributed among insect populations and species (e.g., both infected and uninfected populations can be observed in the same species; Werren, 1997). In infected nematode species, all individuals and populations thus far examined are infected (Bandi et al., 2001). *Wolbachia* strains that infect arthropods can tolerate the cellular environment of diverse hosts, as demonstrated by studies with microinjection in various host cells (Boyle et al., 1993; Braig et al., 1994). In addition, there is evidence for horizontal transmission of *Wolbachia* in arthropods, and the same host may be infected by different strains of *Wolbachia* belonging to different genomic groups (Werren, 1997; Jamnongluk et al., 2002). Horizontal transmission and

multiple infections have not been documented for nematode-infecting *wolbachiae* (Bandi et al., 1999, 2001; Casiraghi et al., 2001a).

Wolbachia does not usually cause obvious damage to its host cell. Nevertheless, a variant strain detected in *Drosophila melanogaster* showed massive proliferation in adult cells leading to widespread degeneration of tissues followed by early death of the insect (Min and Benzer, 1997). In addition, the male-killing *Wolbachia* strains present in arthropods (see below) are obviously pathogenic to male embryos (Hurst et al., 1999).

In arthropods, *Wolbachia* infection is usually associated with alterations in host reproduction, which include killing of male embryos, induction of parthenogenesis, feminization of genetic males, and cytoplasmic incompatibility (CI) (reviewed in Werren, 1997; Stouthamer et al., 1999; Stevens et al., 2001). It must be noted that *Wolbachia* is transmitted to the offspring by females, whereas males are usually not involved in transmission. All the reproductive alterations effected by *Wolbachia* in arthropods are interpreted as having the overall effect of increasing the transmission rate of *Wolbachia* (Werren, 1997). For example, parthenogenesis and feminization cause a *Wolbachia*-infected female to generate more female offspring, which will in turn transmit the bacterium. In CI, embryonic death is observed after mating between males that are infected by certain strains of *Wolbachia* and females that are either uninfected or infected with an incompatible *Wolbachia* strain. i.e., males infected by *Wolbachia* do not

TABLE BXII.α.42. Prevalence of *Wolbachia* in arthropods and filarial nematodes^a

Host taxa	<i>Wolbachia</i> prevalence	Reference
<i>Arthropods:</i>		
Arthropoda	76%	Jeyaprakash and Hoy, 2000
Arachnida	(1/2)	
Insecta	(47/61)	
Arthropoda	18.4%	Werren and Windsor, 2000
Arachnida	(1/12)	
Insecta	(28/145)	
Arthropoda	16.5%	Werren et al., 1995a
Arachnida	(0/3)	
Insecta	(26/154)	
Insecta (Hymenoptera and Diptera)	26%	Cook and Butcher, 1999
Insecta (Hymenoptera)	59.30%	Plantard et al., 1999
Insecta (Hymenoptera)	50%	Wenseleers et al., 1998
Crustacea (Isopoda)	30%	Bouchon et al., 1998
Insecta	21.70%	West et al., 1998
Insecta (Diptera)	89%	Bandi et al., 1997
<i>Filarial nematodes:</i>		
<i>Acanthocheilonema viteae</i>	No <i>Wolbachia</i> observed	McLaren et al., 1975; Bandi et al., 1998; Hoerauf et al., 1999
<i>Brugia malayi</i>	<i>Wolbachia</i> observed	Bandi et al., 1998; Taylor et al., 1999
<i>Brugia pahangi</i>	<i>Wolbachia</i> observed	Bandi et al., 1998; Taylor et al., 1999
<i>Dirofilaria immitis</i>	<i>Wolbachia</i> observed	Sironi et al., 1995
<i>Dirofilaria repens</i>	<i>Wolbachia</i> observed	Bandi et al., 1998
<i>Litomosoides sigmodontis</i>	<i>Wolbachia</i> observed	Bandi et al., 1998; Hoerauf et al., 1999
<i>Mansonella ozzardi</i>	<i>Wolbachia</i> observed	Casiraghi et al., 2001a
<i>Onchocerca flexuosa</i>	No <i>Wolbachia</i> observed	Henkle-Dührsen et al., 1998; Plenge-Bönig et al., 1995
<i>Onchocerca gibsoni</i>	<i>Wolbachia</i> observed	Bandi et al., 1998
<i>Onchocerca gutturosa</i>	<i>Wolbachia</i> observed	Bandi et al., 1998
<i>Onchocerca lupi</i>	<i>Wolbachia</i> observed	Egyed et al., 2002
<i>Onchocerca ochengi</i>	<i>Wolbachia</i> observed	Bandi et al., 1998
<i>Wuchereria bancrofti</i>	<i>Wolbachia</i> observed	Bandi et al., 1998; Taylor et al., 1999

^aData from Taylor and Hoerauf (1999), Bandi et al. (2001), and Stevens et al. (2001). For filarial nematodes, the *Wolbachia*-infected species included in the table are those for which positive *Wolbachia* PCR and *Wolbachia* gene sequences have been obtained; the *Wolbachia*-negative filarial species included in the table (*A. viteae* and *O. flexuosa*) are those for which the evidence for the absence of intracellular bacteria or *Wolbachia* has been reported in independent experimental studies based on different approaches (e.g., electron microscopy and PCR); for other filarial species that may be either infected or uninfected by *Wolbachia*, see the tables published by Taylor and Hoerauf (1999) and Bandi et al. (2001).

transmit it, but they sterilize those females that do not carry *Wolbachia* or those that carry a different compatibility type of *Wolbachia*. This reduction in the fitness of uninfected females implies an increase of the fitness of infected ones, thereby favoring the spread of the CI-inducing *Wolbachia* in the host population.

The molecular mechanisms of the reproductive alterations caused by *Wolbachia* are not known, even though the events leading to phenomena like CI have been described at the cytological level (e.g., Tram and Sullivan, 2002). In addition to the effects on arthropod reproduction, there is one case of a wasp (*Asobara tabida*) in which *Wolbachia* is required for oogenesis (Dedeine et al., 2001). *Wolbachia* has also been shown to rescue a deleterious genetic mutation that affects oogenesis in *Drosophila melanogaster* (Starr and Cline, 2002). Moreover, the number of *Wolbachia* cells in filarial nematodes has been shown to be positively associated with longevity (Taylor, personal communication).

There are different lines of evidence implicating *Wolbachia* in the pathogenesis of filariasis and in the side effects of anti-filarial therapy. In particular, extracts of filariae harboring *wolbachiae* stimulate human and mouse monocytes to produce pro-inflammatory cytokines and other mediators of inflammation (Brattig et al., 2000; Taylor et al., 2000), and they also attract granulocytes to the cornea in an animal model of river blindness, with thickening and opacity of the corneal stroma (Saint André et al., 2002). Both corneal alterations and monocyte stimulation have been shown to be dependent on receptors required for the host cell response to lipopolysaccharide (LPS). In addition, monocyte stimulation is inhibited by an LPS antagonist/inhibitor, but not by heat treatment of the filarial extract (Taylor et al., 2000). Thus, there is overall evidence that filariae that harbor *Wolbachia* strains also contain LPS, which might play an important role in the immunopathogenesis of filariasis. The presence of LPS in these filariae is supported by a positive *Limulus* amebocyte lysate assay on filarial extracts (Brattig et al., 2000; Taylor et al., 2000). However, the LPS of *Wolbachia* has not yet been purified. The fact that *Wolbachia* actually interacts with the host immune system is further documented by the presence of antibodies against the *Wolbachia* surface protein (WSP) in animals infected with filarial nematodes (Bazzocchi et al., 2000b; Punkosdy et al., 2001). In addition, real-time PCR has allowed quantification of the release of *Wolbachia* DNA after microfilaricidal treatment of patients affected by onchocerciasis. There is a correlation between the PCR signal for *Wolbachia*, the release of mediators of inflammation, and the side effects of therapy (Keiser et al., 2002). It must be noted that post-treatment reactions in filariasis partially resemble those observed in acute bacteremia, both at the clinical level and in terms of markers of inflammation (Haarbrink et al., 2000; Keiser et al., 2002).

Susceptibility of *W. pipientis* to tetracycline was first demonstrated by addition of 17-50 µg/ml of tetracycline to the diet of larvae, resulting in the elimination of *Wolbachia* from the insects (Weiss et al., 1984). Studies of antibiotic susceptibility of *W. pipientis* were extended by studies of the susceptibility of the O'Neill strain in the Aa23 cell line to five antibiotic agents (Hermans et al., 2001) (Table BXII.α.43). *In vivo* studies in filarial nematode models have also demonstrated the activity of rifampicin and doxycycline and the inactivity of ciprofloxacin against *Wolbachia* (Hoerauf et al., 2000a; Towson et al., 2000). Treatment with tetracycline and tetracycline derivatives has also been shown to have detrimental effects on filariae that harbor *Wolbachia*

TABLE BXII.α.43. Effects of antibiotics on the O'Neill strain of *Wolbachia pipientis*^{a, b}

Antibiotic	MIC (mg/l)	MBC (mg/l)
Doxycycline	0.0625	0.25
Oxytetracycline	4	1
Rifampicin	0.0625	2
Ciprofloxacin	>8	>8
Penicillin	>256	>256

^aData from Hermans et al. (2001).

^bMIC, minimal inhibitory concentration; MBC, minimal bacteriocidal concentration.

(Bandi et al., 1999; Hoerauf et al., 1999, 2000a, b; McCall et al., 1999; Langworthy et al., 2000; Towson et al., 2000; Casiraghi et al., 2002). These detrimental effects include inhibition of worm development, infertility, and adulticidal effects (reviewed in Bandi et al., 2001). Since tetracycline treatment has no detrimental effect on the *Wolbachia*-free filarial worm *A. viteae*, it is assumed that, in infected species, *Wolbachia* is needed by the host nematode (Hoerauf et al., 1999; McCall et al., 1999). The discovery that filarial nematodes are susceptible to antibiotics has opened new prospects for the control of filariasis: clinical trials have already demonstrated the potential utility of doxycycline for the control of river blindness (Hoerauf et al., 2000b).

ENRICHMENT AND ISOLATION PROCEDURES

The sole isolation of *W. pipientis* reported to date is that of cells grown successfully in an Aa23 cell line from *Aedes albopictus* mosquitoes. The cell line was established as described by Tesh and Modi (1983). These cells (both infected ones and ones freed of *W. pipientis* by antibiotic treatment) are routinely grown at 28°C in a mixture (1:1 v/v) of Mitsuhashi-Maramorosh insect medium and Schneider's insect medium supplemented with 10–15% bovine fetal serum. Cells are grown in 25-cm³ flasks containing 5 ml of medium. The cells are passaged every week by shaking the flask and centrifuging the resulting cell suspension at low speed. The cell pellet is resuspended in the same amount of medium, and a new flask is seeded with 20% of the resuspended cells. For production of large amounts of bacteria, infected cells from one flask may be harvested every five days and inoculated into three cell culture flasks with fresh medium. The O'Neill strain can be propagated on human embryonic lung (HEL) fibroblast monolayers (CCL-137, American Type Culture Collection, Rockville, MD) at 28 and 37°C under previously described conditions (Raoult et al., 2001). The strain can also be propagated in C6/36—(CRL-1660, ATCC)—another mosquito cell line (from *A. albopictus*) grown in Leibowitz-15 medium with L-glutamine and L-amino acids, 5% (v/v) fetal bovine serum and 2% (v/v) tryptose phosphate at 28°C (Fenollar, La Scola, Taylor, and Raoult, unpublished data).

MAINTENANCE PROCEDURES

The O'Neill strain is preserved by rapid freezing and storage at –80°C.

DIFFERENTIATION OF THE GENUS *WOLBACHIA* FROM OTHER GENERA

Wolbachia are differentiated from *Anaplasma*, *Ehrlichia*, and *Neorickettsia* because they do not form morulae and because they infect only arthropods and filarial nematodes, not mammals. They are also distinguished by their 16S rRNA gene sequence.

TAXONOMIC COMMENTS

A phylogenetic analysis of the 16S rRNA gene clearly assigned *W. pipientis* to the *Alphaproteobacteria* and revealed a close relationship to the genera *Ehrlichia*, *Anaplasma* and *Neorickettsia* (Fig. BXII.α.45) (O'Neill et al., 1992). This study showed that *W. pipientis sensu stricto* (i.e., the bacterium present in the ovaries of *C. pipiens*) formed a monophyletic clade with other insect-associated microorganisms, and the authors suggested that this analysis supported the provisional "classification of these bacteria as members of the same species" (O'Neill et al., 1992). Further analyses based on the 16S rRNA gene and other genes identified other bacteria found in arthropods and in filarial nematodes as close relatives of *W. pipientis* (Rousset et al., 1992; Stouthamer et al., 1993; Werren et al., 1995b; Bandi et al., 1998; Vandekerckhove

et al., 1999; Lo et al., 2002). All these bacteria form a coherent, monophyletic clade composed of at least six different main clusters (Fig. BXII.α.48), which have been indicated as supergroups A–F (Lo et al., 2002). In agreement with the opinion expressed by O'Neill et al. (1992), it has been suggested that the six supergroups be provisionally regarded as belonging to the sole valid species of the genus, *W. pipientis*, until formal proposals are made to elevate these clusters at the species rank (Bandi et al., 2001; see also discussion in Dumler et al., 2001).

At a finer taxonomic scale, a system based on the level of similarity in the *wsp* gene sequence has been proposed for strain grouping (Zhou et al., 1998). However, evidence for recombination between *Wolbachia* strains of supergroups A and B has raised questions about the usefulness of strain assemblages based

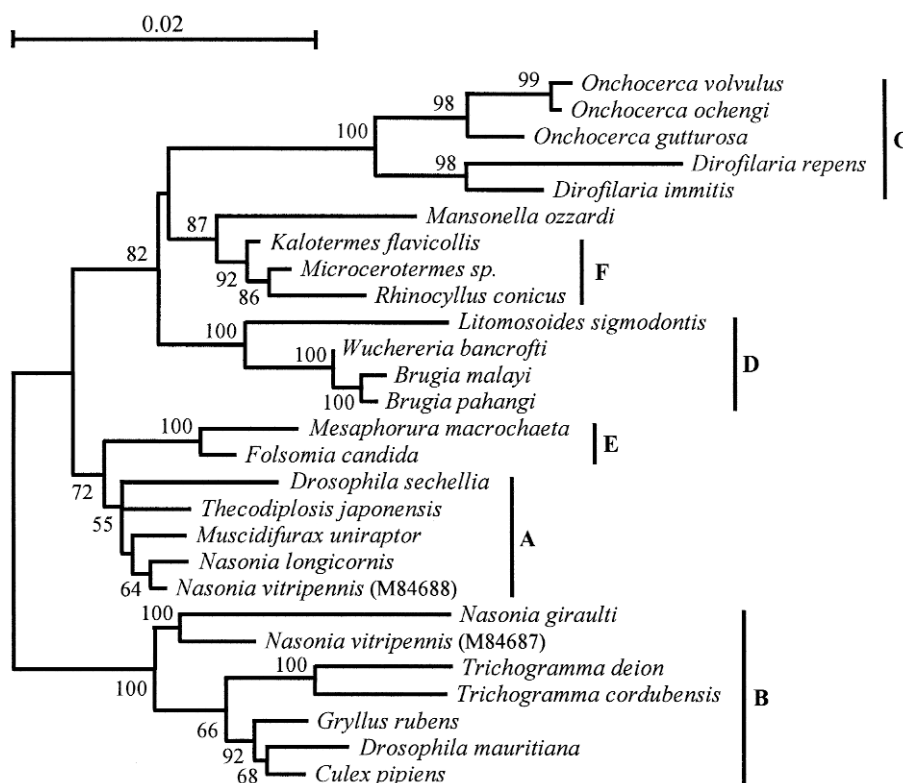


FIGURE BXII.α.48. Unrooted phylogenetic tree showing the six supergroups of *Wolbachia pipientis* (A–F) Lo et al., 2002. Names at the terminal nodes are those of the host species. The scale bar indicates distance in substitutions per nucleotide. Numbers at the nodes are the bootstrap confidence values based on 100 replicates (values below 50% are not indicated). Supergroups A, B, E, and F are found in arthropods. Supergroups C and D are found in filarial nematodes. The positioning of the *Wolbachia* found in the filarial nematode *Mansonella ozzardi* as a sister group of F *wolbachiae* is still to be evaluated, since only the 16S rRNA gene is available for this symbiont. The tree (neighbor-joining method; Kimura correction) is based on the gene coding for the 16S rRNA. This tree includes *Wolbachia* sequences derived from: *Nasonia vitripennis*, GenBank accession number M84688; *Mesaphorura macrochaeta*, GenBank accession number AJ422184; *Gryllus rubens*, GenBank accession number U83092; *Muscidifurax uniraptor*, GenBank accession number L02882; *Rhinocyllus conicus*, GenBank accession number M85267; *Nasonia longicornis*, GenBank accession number M84691; *Nasonia vitripennis*, GenBank accession number M84687; *Nasonia giraulti*, GenBank accession number M84689; *Trichogramma cordubensis*, GenBank accession number L02883; *Trichogramma deion*, GenBank accession number L02884; *Culex pipiens*, GenBank accession number X61768; *Drosophila mauritiana*, GenBank accession number U17060; *Drosophila sechellia*, GenBank accession number U17059; *Folsomia candida*, GenBank accession number AF179630; *Thecodiplosis japonensis*, GenBank accession number AF220604; *Kaloterms flavicollis* Y11377; *Microcerotermes* sp., GenBank accession number AJ292347; *Brugia pahangi*, GenBank accession number AJ012646; *Brugia malayi*, GenBank accession number AJ010275; *Wuchereria bancrofti*, GenBank accession number AF093510; *Onchocerca ochengi*, GenBank accession number AJ010276; *Dirofilaria immitis*, GenBank accession number Z49261; *Mansonella ozzardi*, GenBank accession number AJ279034; *Dirofilaria repens*, GenBank accession number AJ276500; *Litomosoides sigmodontis*, GenBank accession number AF069068; *Onchocerca volvulus*, GenBank accession number AF069069; *Onchocerca gutturosa*, GenBank accession number AJ276498.

on single gene sequences (Jiggins et al., 2001; Werren and Bartos, 2001).

The inference of phylogenetic relationships among strains of *Wolbachia* and the description of the six supergroups have been based on the analyses of 16S rRNA and *ftsZ* genes (Werren et al., 1995b; Bandi et al., 1998; Lo et al., 2002). At the level of supergroup definition, analyses of other genes (*wsp*; *groESL*) have led to results that are generally congruent with those based on 16S rDNA and *ftsZ* genes (Masui et al., 1997; Bazzocchi et al., 2000a). However, most of the phylogenetic trees thus far published for *Wolbachia* are actually unrooted (e.g., Werren et al., 1995a; Bandi et al., 1998; Zhou et al., 1998). In the absence of a reliable rooting for the overall phylogeny of *Wolbachia* and of suitable outgroup sequences, the relationships among the six supergroups are indeed not yet established. The possibility that one of the supergroups is paraphyletic cannot be excluded.

Supergroups A, B, E, and F contain the wolbachiae found in arthropods, whereas supergroups C and D contain the wolbachiae of filarial nematodes. One possible exception is the *Wolbachia* strain harbored by the filarial nematode *Mansonella ozzardi*; this strain appears to be more closely related to members of supergroup F (Lo et al., 2002). In supergroups A and B the phylogeny of *Wolbachia* is not always congruent with the host phylogeny (O'Neill et al., 1992; Werren et al., 1995b); this find-

ing suggests that *Wolbachia* can be horizontally transmitted among arthropod hosts (Werren, 1997). In supergroups C and D the phylogeny of *Wolbachia* is consistent with the phylogeny of the host nematodes, which suggests strict vertical transmission of the symbionts (Casiraghi et al., 2001b). In a single infected nematode species, all the individuals thus far examined have been shown to harbor *Wolbachia* strains with identical *wsp* gene sequences, while *Wolbachia* strains from different nematode species possess differences in this gene (Bazzocchi et al., 2000b). This result supports the hypothesis of strict vertical transmission and suggests that the association with *Wolbachia* is species-specific in filarial nematodes.

FURTHER READING

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- Stouthamer, R., J.A.J. Breeuwer and G.D.D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 53: 71–102.
- Werren, J.H. 1997. Biology of *Wolbachia*. *Annu. Rev. Entomol.* 42: 587–609.

List of species of the genus *Wolbachia*

1. *Wolbachia pipientis* Hertig 1936, 472^{AL}

pi.pi.en'tis. M.L. n. *pipiens* specific epithet of the host mosquito, *Culex pipiens*; M.L. gen. n. *pipientis* of *pipiens*.

The characteristics are as described for the genus.

The mol% G + C of the DNA is: unknown.

Type strain: no type strain.

Additional Remarks: The sole available strain for *in vitro* growth is O'Neill strain (see above).

Genus *Incertae Sedis* V. *Aegyptianella* Carpano 1929, 12^{AL}

YASUKO RIKIHISA AND JULIUS P. KREIER

Ae.gyp'ti.a.nel'la. dim. ending -ella M.L. fem. dim. n. *Aegyptianella* named after Egypt where the organism was described in 1929.

In blood smears stained by Romanowsky methods, the inclusions appear in erythrocytes as purple intracytoplasmic bodies of 0.3–4 µm in size. By electron microscopy, each inclusion contains between 1 and 26 pleomorphic cocci with trilaminar outer membranes. They are obligate parasites of domestic and wild birds. Transmitted by ticks.

The mol% G + C of the DNA is: not known.

Type species: *Aegyptianella pullorum* Carpano 1929, 12.

FURTHER DESCRIPTIVE INFORMATION

Phylogeny By 16S rRNA gene and *groEL* operon sequence analysis, *Aegyptianella pullorum* belongs to the family *Anaplasmataceae* (Rikihisa et al., 2003). Identities of 16S rRNA genes and *groEL* operon sequences of *A. pullorum* to those of *Anaplasma* species were 92.7–93.3% and 71.4–73.3%, respectively. Identities of 16S rRNA gene and *groEL* operon sequences of *A. pullorum* to those of *Ehrlichia* species were 86.0–88.2 and 68.3–69.2%, respectively. Identities of 16S rRNA gene and *groEL* operon sequences of *A. pullorum* to those of *Neorickettsia* species were 78.2–79.2% and 40.0–41.5%, respectively.

Cell morphology In blood smears stained with Giemsa, the inclusions appear in the host's erythrocytes in a variety of forms: compact, round or oval, ring- or horseshoe-shaped, polygonal or polymorphic. They are violet-reddish in color, with a diameter of 0.3–4.0 µm. In larger inclusions, clearly defined small cocci of 0.25–0.4 µm resembling *Anaplasma* sp. can be distinguished by electron microscopy. The organisms reproduce by binary fission. These bacteria may also be found free in the plasma and in phagocytic cells. Organisms in phagocytic cells are probably phagocytosed and not growing, but undergoing digestion (Gothe, 1967b, 1971).

Fine structure In erythrocytes, the inclusions are separated from the erythrocyte cytoplasm by a single membrane presumably of erythrocyte origin. The bacterial cytoplasm contains ribosomes and DNA strands. The organisms are enveloped in a trilaminar outer membrane (Gothe, 1967a, 1971; Bird and Garnham, 1969; Castle and Christensen, 1985) (Fig. BXII.α.49). Scanning electron microscopy of infected erythrocytes revealed that the mode of entrance of organisms into erythrocytes may be

endocytosis followed by formation of an erythrocytic vesicle. The exit of the organisms from parasitized erythrocytes may be the reverse of the invasive mechanism, an exocytosis. Generally, however, the affected erythrocytes are injured by the parasites, resulting in release of the parasites into the plasma by host cell lysis (Gothé and Burkhardt, 1979).

Cultural characteristics and antibiotic susceptibility Multiplication of *A. pullorum* has not been observed to occur in cell-free media or in tissue cultures. Attempts at continuous propagation of the organism in chicken embryos have not been successful (Gothé, 1971). Only broad-spectrum antibiotics of the tetracycline series, dithiosemicarbazones, and pleuromutilins have a bacteriocidal efficacy with a significant chemotherapeutic influence on the course of the infection in chickens (Gothé, 1971; Gothé and Mieth, 1979).

Ecology Chickens are naturally infected with *A. pullorum* by the ticks *Argas (Persicargas) persicus*, *A. (P.) walkerae*, *A. (P.) sanchezi*, and *A. (P.) radiates*. The tick is a biological vector. Experimental infection can be achieved by subcutaneous, intramuscular, intravenous, and intraperitoneal inoculation of infected blood or by scarification followed by application of the infected blood to the scarified area. In addition to infection of chickens, natural infections have been described in geese, ducks, quail, and ostriches. Wild birds that have been experimentally infected are *Turtur erythrophrys* and *Balearica pavonina* (Curasson and Andrjesky,

1929), *Turtur senegalensis*, *Milvus aegyptiacus*, and *Vidua principalis* (Curasson, 1938).

Infection is transstadial in ticks (Gothé, 1967c, 1971; Hadani and Dinur, 1968). Transovarial transmission has also been observed (Hadani and Dinur, 1968; Gothé, 1971).

MAINTENANCE PROCEDURES

The infectivity of *A. pullorum* in chicken blood can be preserved up to nearly 7 years by storage in liquid nitrogen (Raether and Seidenath, 1977). Cryopreservation does not affect the ability of the parasites to propagate in the vector tick *Argas (Persicargas) walkerae* (Gothé and Hartmann, 1979).

DIFFERENTIATION OF THE GENUS *AEGYPTIANELLA* FROM OTHER GENERA

Characteristics useful for differentiating *Aegyptianella* from the other members of the family *Anaplasmataceae* are provided in the key to this family (see Table BXII.α.33 of the chapter describing the family *Anaplasmataceae*).

TAXONOMIC COMMENTS

At present several species have been described in the genus *Aegyptianella* including those bacteria infecting erythrocytes of poikilotherms (Gothé, 1978). Detailed morphological and biochemical investigations have only been carried out with *A. pullorum*.

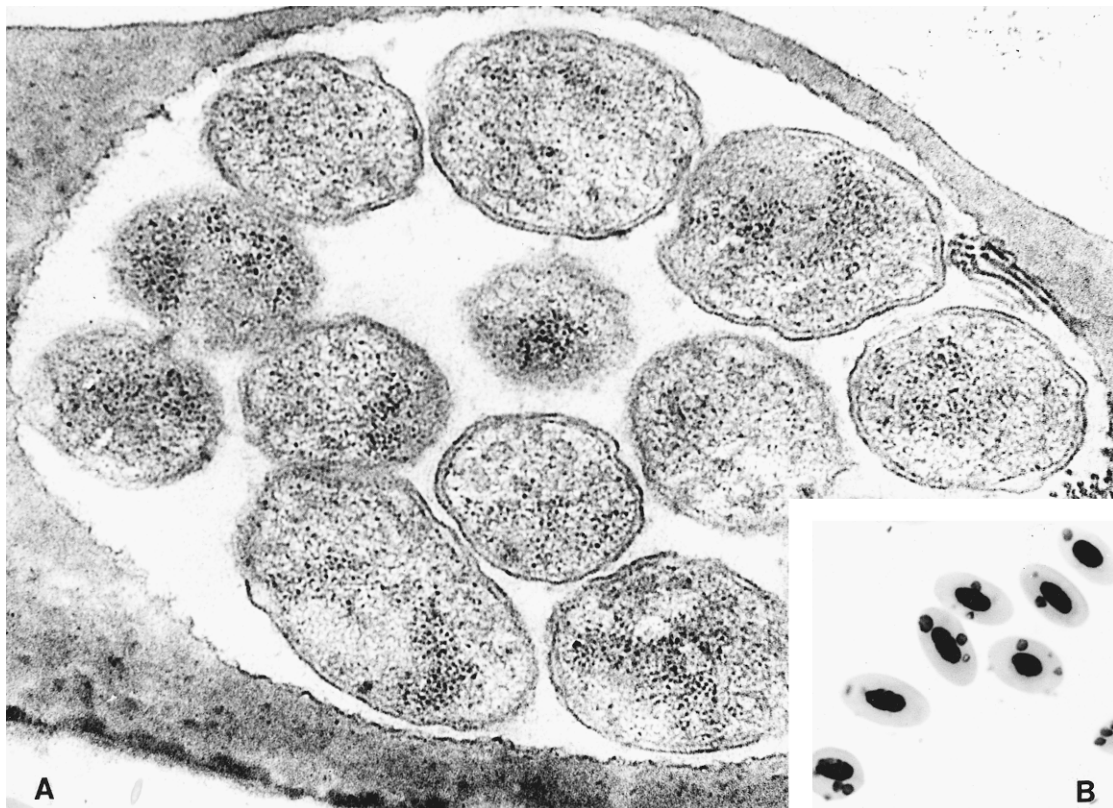


FIGURE BXII.α.49. *Aegyptianella pullorum*. A, ultrathin section of an infected erythrocyte, showing an inclusion body containing 12 parasites ($\times 90,000$). B, intraerythrocytic inclusion bodies stained by the Giemsa method. (Reproduced with permission from R. Gothé, *Zeitschrift für Parasitenkunde*, 29: 119–129, 1967, ©Springer-Verlag, Berlin.)

Electron microscopy studies have been performed for “*Aegyptianella botuliformis*”, “*Aegyptianella ranarum*”, and “*Aegyptianella bacterifera*”, which show that these three spp. are different from *A. pullorum*. Other species have been described but, except for “*A. ranarum*”, neither their true identity nor their relationship to *A. pullorum* has been established (see *Species incertae sedis*).

FURTHER READING

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DIFFERENTIATION OF THE SPECIES OF THE GENUS AEGYPTIANELLA

The following species are tentatively classified in the genus *Aegyptianella*; genetic studies are required to learn how similar they are. Except for *A. pullorum*, these species have not been formally

described and therefore do not presently have standing in nomenclature. Characteristics are listed in Table BXII.α.44.

List of species of the genus Aegyptianella

1. ***Aegyptianella pullorum*** Carpano 1929, 12^{AL}.
pullor'um. L. gen. pl. n. *pullorum* of young fowls.
The characteristics are as described for the genus.
The mol% G + C of the DNA is: unknown.
Type strain: No culture isolated.
Additional Remarks: At the time of publication, the only available 16S rRNA gene sequence for *A. pullorum* was AY125087.

Species Incertae Sedis

1. ***Aegyptianella bacterifera*** Barta, Boulard and Desser 1989, 14 (“*Cytamoeba bacterifera*” Labbé 1894, 104.)
bac.te.ri'fe.ra. Gr. dim. n. *bacterium* a small rod; L. suffix *-fer* (-*ferus*), from L. v. *fero* to bear; M.L. adj. *bacterifera* bearing small rods.
Up to 12 rod-shaped organisms that are $2.3\text{--}4.9 \times 0.5 \mu\text{m}$ are found in membrane-bound inclusions of erythrocytes of *Rana esculata* in Corsica and *R. nigromaculata* in China.
2. ***Aegyptianella botuliformis*** Huchzermeyer, Horak, Putterill and Earle 1992, 100.
botuli'formis. L. gen. n. *Botulus* sausage; L. n. *forma* shape, form; M.L. adj. *botuliformis* sausage-shaped.
In small inclusions it resembles *A. pullorum*, but in larger inclusions organisms are sausage-shaped with up to eight organisms tightly packed in an inclusion. It does not infect

chickens but produces a high and long-lasting parasitemia in guinea fowl. Infection is associated with *Amblyomma hebraeum* or *A. marmoreum* infestation.

Type strain: no culture isolated.

The mol% G + C of the DNA is: unknown.

3. ***Aegyptianella carpani*** Battelli 1947, 212.
Found in the snake *Naia nigricollis*.
4. ***Aegyptianella elgonensis*** Mutinga and Dipeolu 1989, 410.
Found in the lizards collected near Mt. Elgon.
5. ***Aegyptianella ranarum*** Desser 1987, 53.
rana'rum. L. n. *rana* frog; M.L. adj. *ranarum* of frog.
Up to 120 rod-shaped organisms that are 150–200 nm \times 1–1.7 μm are found in membrane-bound inclusions of erythrocytes of bullfrogs (*Rana catesbeiana*), green frogs (*R. clamitans*), and mink frogs (*R. septentrionalis*).
Identity of the 16S rRNA gene sequence of “*A. ranarum*” (AY208995) to that of *A. pullorum* (AY125087) is 60.9%, showing that “*A. ranarum*” does not belong to the genus *Aegyptianella* or even to the family *Anaplasmataceae* (Rikihisa, unpublished results).
6. ***Sogdianella moshkovskii*** (Laird and Lari 1957) Schurenkova 1938, 936 (“*Babesia moshkovskii*” Laird and Lari 1957, 794.)
Found in various wild birds.
7. ***Tunetella emydis*** Brumpt and Lavie 1935, 548.
Found in the tortoise *Emys leprosa*.

TABLE BXII.α.44. Differential characteristics of the species of the genus *Aegyptianella*

Characteristic	<i>A. pullorum</i> ^a	“ <i>A. bacterifera</i> ” ^b	“ <i>A. botuliformis</i> ” ^c	“ <i>A. ranarum</i> ” ^d
Morphology	Cocci	Rods	Rods	Rods
Host	Chicken, goose, duck, ostrich, quail, wild turkey	Frog (<i>Rana nigromaculata</i> , <i>R. esculata</i>)	Helmeted guinea fowl (<i>Numida meleagris</i>)	Frog (<i>Rana catesbeiana</i> , <i>R. septentrionalis</i> , <i>R. clamitans</i>)
Location	North Africa, North America	China, Corsica	South Africa	Canada
Possible vector	<i>Argas walkerae</i> , <i>A. sanchezi</i> , <i>A. radiatus</i>	Unknown	<i>Amblyomma hebraeum</i> , <i>A. marmoreum</i>	Leech (<i>Batrachobdella picta</i>)

^aGothé and Kreier, 1984; Castle and Christensen, 1985.

^bWerner, 1993; Desser and Bartha, 1989.

^cHuchzermeyer et al., 1992.

^dDesser, 1987.

Family III. **Holosporaceae** *fam. nov.*

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Ho.lo' spo.ra.ce.ae. Gr. fem. adj. *holos* whole, complete; Gr. n. *sporus* seed; M.L. n. *spora* spore; M.L. fem. n. *Holospora* whole spore; *-ceae* ending to denote family; *Holosporaceae* family of *Holospora* bacteria.

Bacteria are present specifically in the **micronucleus or macronucleus** of *Paramecium* species. Exists in two forms: the **reproductive form** is a short rod $1.0\text{--}3.0 \times 0.5 \mu\text{m}$. It undergoes binary fission and may give rise to the **long infectious form**, measuring $5.0\text{--}20.0 \mu\text{m}$ with rounded or tapered ends, that can infect *Paramecium* and become established in the nucleus. The infectious form has a voluminous periplasm filled with fine, granular material with a less electron-dense pale tip, called the special tip. The condensed-looking bacterial protoplasm of the infectious form contains ribosomes and mesosome-like structures. The cytoplasm is polarly positioned opposite to the special tip in one half of the cell (Fig. BXII.α.50). The cytoplasm stains with DNA-specific dyes. Gram-negative, nonmotile, obligate symbiont. No toxic effects of *Holospora*-bearing paramecia on paramecia lacking the symbiont were observed. Paramecia can outgrow *Holospora* species, but reinfection occurs readily; mass cultures show up to 100% infection.

Type genus: Holospora (ex Hafkine 1890) Gromov and Ossipov 1981, 351.^{VP}

FURTHER DESCRIPTIVE INFORMATION

The family currently contains only the single genus *Holospora*. In 1890 the genus *Holospora*, containing three species, was described by Hafkine (1890). Nine species have now been identified, but only four of them were validly published (see list of the species of the genus *Holospora*). Holosporas share a number of unique features. All invade the nuclei of *Paramecium* (Fig. BXII.α.51) (Ciliophora, Protozoa) (Hafkine, 1890; Gromov and Ossipov, 1981; Görtz, 1983, 1986; Fokin, 1989). They are host specific, infecting only certain species of the genus *Paramecium*. All species show a developmental cycle with an infectious and a reproductive form (Fig. BXII.α.52). Only the infectious form is infectious and even short infectious forms were found to be fully infectious (Kawai and Fujishima, 2000). The infectious form shows a unique cellular organization (Fig. BXII.α.50).

Holosporas are truly parasitic. Infectious forms of the bacteria are regularly released by the host cells into the surrounding medium and may infect new host cells. If the macronucleus of a paramecium (see Fig. BXII.α.53 for the organization of the ciliate cell) is infected, this may affect its growth rate and under unfavorable conditions even kill the cell. Under favorable conditions, host cells are not damaged, and some natural populations have been found stably infected for years with infection rates of close to 100%.

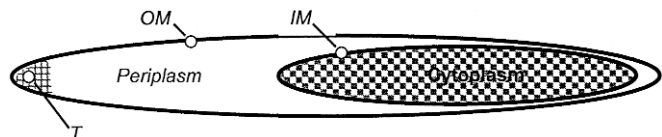


FIGURE BXII.α.50. Diagram of the infectious form of *H. obtusa*. The cytoplasm is condensed and shifted towards one pole. The electron-dense material of the periplasm fills more than half of the cell, with less electron-dense material (*T*) at the pole opposite to the cytoplasm. *IM*, inner membrane; *OM*, outer membrane.

Micronuclear-specific infections are different. In an infection of the micronucleus with *Holospora elegans*, host cell conjugation (sexual propagation) was no longer successful (Görtz and Fujishima, 1983) and exconjugant cells were not viable. Apparently, new macronuclei were not functional. The inhibition of successful conjugation renders the host cells genetically dead.

The individual host cell is not necessarily killed by infection with *Holospora*. Rather, bacteria and host appear to be well adapted. In starving *Paramecium* or after inhibition of host protein synthesis, most bacteria differentiate into the infectious form (Fujishima, 1993), which is a resting stage and does not multiply. In addition, paramecia seem to have mechanisms to cure themselves of endonuclear symbionts. Skoblo et al. (1990), Ossipov et al. (1993), and Fokin and Skovorodkin (1991, 1997) have discovered that *Holospora* may be synchronously and completely lysed in the host nuclei of certain strains of *Paramecium* after an infection. This lysis may be due to an unknown defense mech-

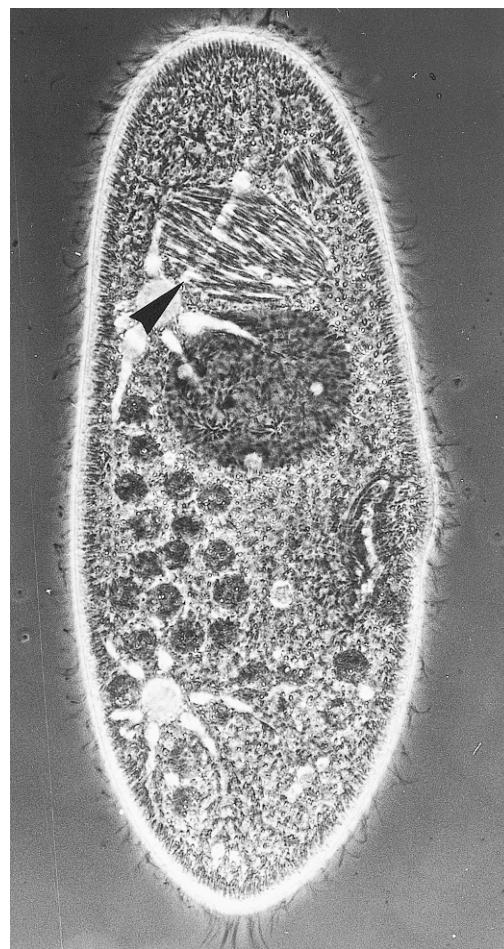


FIGURE BXII.α.51. *Holospora elegans* in the micronucleus (arrow) of *Paramecium caudatum*. Due to the infection, the micronucleus has almost reached the size of the macronucleus.

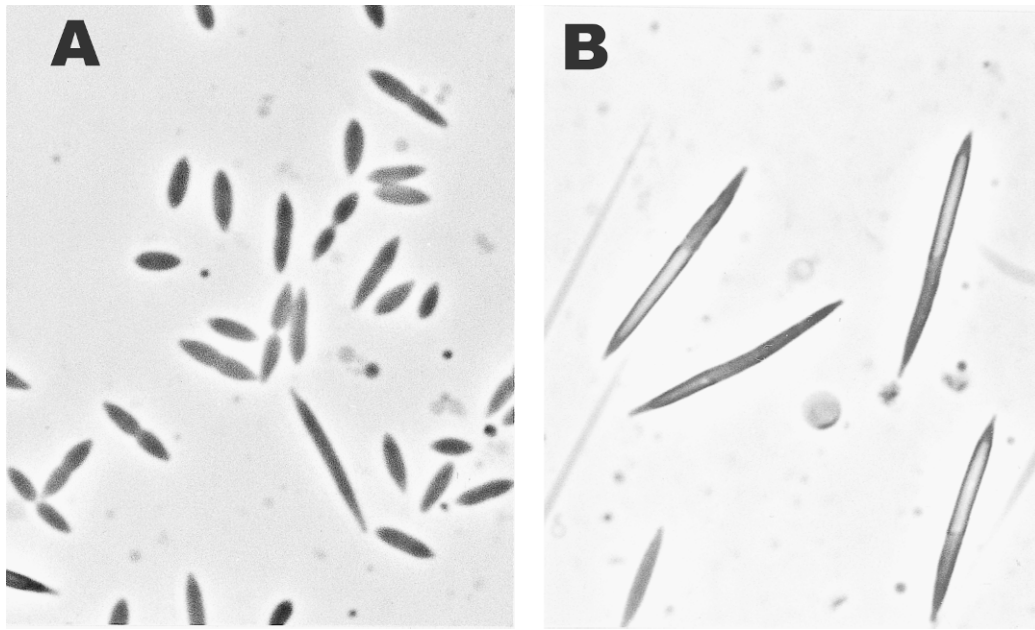


FIGURE BXII.α.52. Reproductive (A) and infectious (B) forms of *Holospora elegans* released from the micronucleus of *Paramecium caudatum*.

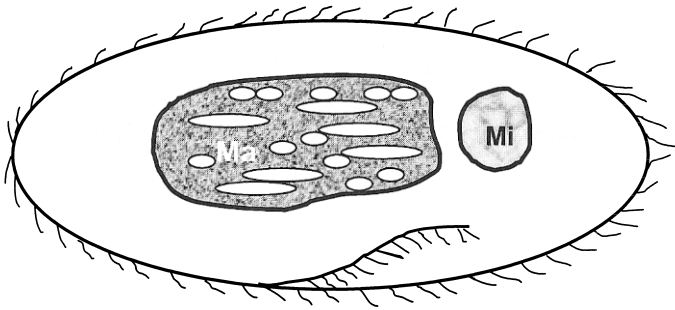


FIGURE BXII.α.53. Diagram of a ciliate cell infected by a *Holospora* species in the macronucleus (schematic representation). *Mi*, micronucleus; *Ma*, macronucleus. The micronucleus is the generative nucleus and transcriptionally inactive. The macronucleus is the somatic nucleus and transcriptionally active. After conjugation, a new macronucleus is developed from the new micronucleus, both deriving from the syncaryon.

anism against intracellular infections evolved in ciliates. After lysis of the bacteria, the host cells are cured and remain viable.

Developmental cycle *Holospora* show a developmental cycle with a specialized infectious form (Figs. BXII.α.50, BXII.α.52). After division of the host cell, only infectious forms are released into the medium, while the reproductive forms remain in the host nuclei. Some reproductive forms may then develop into further infectious forms. The developmental cycle is described in Fig. BXII.α.54.

A new infection may begin with the ingestion of an infectious form. The bacterium leaves the phagosome, is transported through the cytoplasm, and is taken into the host nucleus by fusion of the two membranes of the transport vesicle with those of the nuclear envelope (Görtz and Wiemann, 1989). Transport to the nucleus, and within the nucleus during its division, appears to be mediated by the cytoskeleton of the host cell (Ossipov and Podlipaev, 1977; Görtz and Wiemann, 1989).

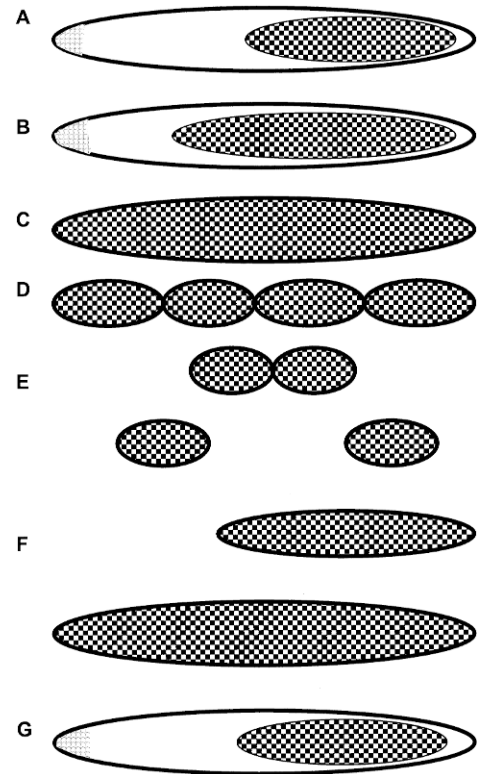


FIGURE BXII.α.54. Diagram of the development of *H. obtusa*. In early stages of invasion, the cytoplasm of the infectious form extends into the periplasmic space, while the volume of periplasm decreases (A, B); after the bacterium arrives in the host nucleus, the whole volume of the cell is occupied by the cytoplasm (C); the cell constricts at several points and divides into small cells (D); by this point, the reproductive form is established and multiplies by binary division (E); reproductive forms may grow out in the host nucleus (F). The resulting long cells develop into the infectious forms by depositing periplasmic material and condensing the cytoplasm (G).

TABLE BXII.α.45. Diagnostic table of *Holospora*^a

Characteristics	<i>H. undulata</i>	<i>H. elegans</i>	<i>H. caryophila</i>	<i>H. obtusa</i>	" <i>H. acuminata</i> "	" <i>H. recta</i> "	" <i>H. curviuscula</i> "	" <i>H. bacillata</i> "	" <i>H. curvata</i> "
Cell morphology of infectious form	Spiral, ends tapered	Straight, ends tapered	Spiral, ends tapered	Straight, ends rounded	Straight rod, ends tapered	Straight, one end tapered	Curved, ends tapered	Straight, ends rounded	Curved
Cell size (μm)	0.8–1 × 10–25	0.8–1 × 10–20	0.2–0.3 × 5–8	0.8–1 × 10–25	0.5–0.6 × 5–8	0.7–1 × 10–15	0.4–0.5 × 4–10	0.7–0.8 × 5–17	0.7–0.9 × 12–20
<i>Host:</i>									
<i>Paramecium biaurelia</i>			+		+		+		
<i>Paramecium bursaria</i>									
<i>Paramecium caudatum</i>	+	+	+	+		+			
<i>Paramecium calkinsi</i>								+	+
<i>Paramecium novaurelia</i>			+						
<i>Paramecium woodruffi</i>								+	
<i>Nucleus:</i>									
Macronucleus			+	+			+	+	+
Micronucleus	+	+			+	+			
<i>Holospora</i> "species group" ^b	I	I	II	I	I	I		II	II

^aFor symbols see standard definitions.

^b*Holospora* species group: I, infectious forms are collected in the connecting piece of the dividing host nucleus and released by the host cell; those species tested were labeled by *in situ* hybridization using a *Holospora*-specific probe (Fokin et al., 1996); II, infectious forms are not collected in the connecting piece of the dividing host nucleus but remain distributed in the dividing nucleus; those species were not labeled by *in situ* hybridization using a *Holospora*-specific probe.

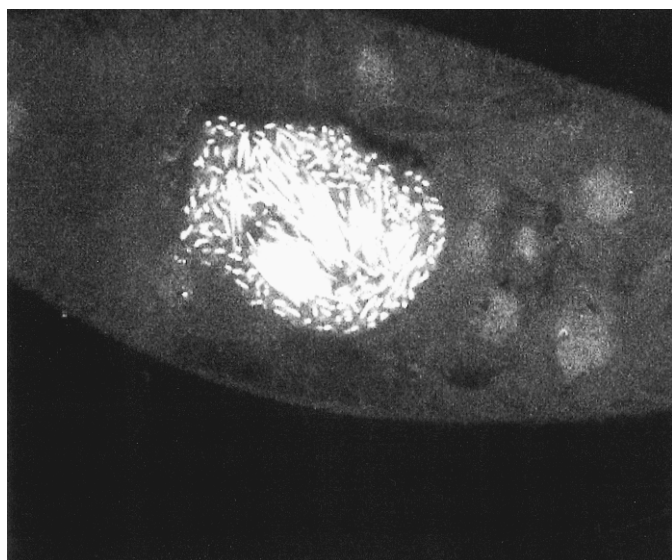


FIGURE BXII.α.55. *Holospora obtusa* in the macronucleus of *Paramecium caudatum*. As a result of hybridization with an *H. obtusa*-specific probe, the bacteria are labeled (Reproduced with permission from S.I. Fokin, European Journal of Protistology Supplement 1, 32: 19–24, 1996, ©Urban & Fischer Verlag GmbH & Co.)

The infectious form has a voluminous periplasm that contains a number of stage-specific proteins, some of which appear to be released during the infection process (Görtz and Wiemann, 1989; Fujishima et al., 1990, 1997; Wiemann and Görtz, 1991; Dohra et al., 1994). Some proteins that were immuno-localized in the periplasm of the infectious form were found on the surface of the bacteria after their ingestion into the phagosome, or associated with the phagosome membrane. This is what would be expected if such proteins were used for communication with host membranes. Released periplasmic proteins could also protect the bacteria against lysosomal enzymes of the host or inactivate such enzymes.

The gene of a small periplasmic protein of 5.4 kDa was sequenced by Dohra et al. (1997). Northern blot hybridization showed that the gene is highly expressed in the intermediate

form, a transitional stage in the development from the reproductive into the infectious form of the bacterium. Amino acid sequence similarities with other polypeptides have not been found (Dohra et al., 1997). It has been suggested that the protein may function in the recognition process in the early phase of infection. Dohra et al. (1998) have also identified a GroEL-like protein in *H. obtusa*. The gene is selectively expressed in the reproductive form.

The infectious form is polarly organized (Figs. BXII.α.50, BXII.α.52) (Dohra and Fujishima, 1999). Half of the cell is occupied by the voluminous periplasm that contains a number of stage-specific proteins. The proteins appear to be released during the infection process. Some proteins were immunolocalized in the periplasm of the infectious form and during the invasion process were found on the surface of the bacteria after their ingestion into the phagosome, associated with the phagosome membrane (Fujishima et al., 1990, 1997; Görtz et al., 1990; Wiemann and Görtz, 1991; Dohra et al., 1994; and unpublished results).

Phylogeny of the *Holosporaceae* *Holospora* species were the first intracellular bacteria in *Paramecium* for which the phylogenetic position was determined (Amann et al., 1991). *H. obtusa*, *H. elegans*, and *H. undulata* belong to the *Alphaproteobacteria*. The closest relative among other symbionts in ciliates was found to be *Caedibacter caryophilus*, and the closest relatives among other bacteria found to date were *Rickettsia* and *Ehrlichia* species (Amann et al., 1991; Springer et al., 1993). It is tempting to regard the striking biology (developmental cycle; host specificity for *Paramecium*, etc.) and the unique morphology of the infectious form as homologous features, proving the close relationship and monophyletic origin of these bacteria. New observations, however, cast doubt on this possibility (Fokin et al., 1996).

The behavior of the infectious forms of certain *Holospora* species, assembling as they do in the connecting piece of the dividing host nucleus, is certainly highly advanced and must be regarded as an apomorphic feature. This behavior ensures that the infectious forms are specifically collected and released by the host cell. *H. caryophila*, *H. bacillata*, and *H. curvata* do not share this feature with the other holosporas. It is not known how the infectious forms of these species leave their host nuclei. They are

either more primitive than the other holosporas, as a quantitative separation of infectious forms and reproductive forms is not observed, or they are not closely related phylogenetically. Two species groups may presently be distinguished in the genus *Holospora* (Table BXII.α.45). It has been hypothesized that the unique behavior of the infectious form to polarly deposit enormous amounts of periplasmic materials could be encoded on a plasmid

or phage genome. However, no plasmid or phage genome has been found (Rautian et al., unpublished results). It is consistent with this observation that the "more advanced" species tested (in which the infectious forms are collected in the separation spindle) gave a positive result after *in situ* hybridization (Fig. BXII.α.55) using an oligonucleotide probe designed for *H. obtusa* (Amann et al., 1991; Fokin et al., 1996).

Genus I. *Holospora* (ex Hafkine 1890) Gromov and Ossipov 1981, 351^{VP}*

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Ho.lo'spo.ra. Gr. fem. adj. *holos* whole, complete; Gr. n. *sporus* seed; M.L. n. *spora* spore; M.L. fem. n. *Holospora* whole spore.

The description of the genus is that of the family. Diagnostic characteristics are given in Table BXII.α.45.

Type species: ***Holospora undulata*** (ex Hafkine 1890) Gromov and Ossipov 1981, 351.

TAXONOMIC COMMENTS

The species listed under "Other Organisms" have not been validly published. They show the typical features of the genus,

namely the expression of a life cycle with infectious and reproductive forms and a strong infectivity for the host nuclei. They share a number of biological features with the validated *Holospora* species and are therefore listed in this chapter. Except for "*H. recta*", where this has been questioned (see below), the bacteria appear to be good species with a distinct host specificity, nucleus-specificity, and clear morphology.

List of species of the genus *Holospora*

1. ***Holospora undulata*** (ex Hafkine 1890) Gromov and Ossipov 1981, 351^{VP}
un.du.la'ta. L. fem. adj. *undulatus* undulated, with waves.

Lives in the micronucleus of *P. caudatum*. Reproductive form short, spindle shaped; infectious form long, spiral shaped. Diagnostic characteristics are given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

Type strain: original description and illustration of Hafkine (1890).

Additional Remarks: Clone M1-48 of *P. caudatum* containing *H. undulata* has been deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, State University of St. Petersburg, Russia.

2. ***Holospora caryophila*** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 141^{VP} (*Cytophaga caryophila* Preer, Preer and Jurand 1974, 156.)
ca.ry.o'phi.la. G. n. *caryum* nut, kernel, nucleus; Gr. adj. *philus* loving, M.L. fem. adj. *caryophila* nucleus loving.

Lives in the macronucleus (Figs. BXII.α.56 and BXII.α.57) of *P. biaurelia* or *P. caudatum*. Reproductive form thin, fusiform rod, 0.3–0.5 × 1.0–3.0 μm; spiral infectious form, 0.2–0.3 × 5–6 μm, tapered ends. Is highly infective to a few stocks of *Paramecium biaurelia* and *P. caudatum*. Known previously as alpha (Preer, 1969).

Recent observations may cast some doubt upon the inclusion of *H. caryophila* in the genus *Holospora*. It was observed that the behavior of *H. caryophila* in dividing host nuclei is different from that of *H. undulata*, *H. elegans*, *H. obtusa*, and "*H. acuminata*". While in those species the in-

fectious forms are collected in the connecting piece of the dividing nucleus and are then released from the nucleus, infectious forms of *H. caryophila* leave the host nucleus by an unknown mode (Fokin et al., 1996). Moreover, in contrast to *H. undulata*, *H. elegans*, and *H. obtusa*, *H. caryophila* is not recognized by the *Holospora* probe in *in situ* hybridization ("*H. acuminata*" not tested; Fokin et al., 1996). Because of these differences to *H. undulata*, *H. obtusa*, and *H. elegans*, *H. caryophila* has been considered to belong to a second group of *Holospora* (Table BXII.α.45).

The mol% G + C of the DNA is: not determined.

Type strain: ATCC 30694.

Additional Remarks: The type strain of *H. caryophila* is in stock 562 of *P. tetraurelia* (ATCC 30694) (Preer et al., 1974).

3. ***Holospora elegans*** (ex Hafkine 1890) Preer and Preer 1982, 141^{VP}
e'le.gans. L. adj. *elegans* choice, elegant.

Lives in the micronucleus of *P. caudatum*. Reproductive form short, spindle-shaped; infectious form long rod with tapered ends. Diagnostic characteristics are given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

Type strain: ATCC 50008.

Additional Remarks: The type strain of *H. elegans* is in stock C101 of *P. caudatum* (ATCC 50008) (Görtz and Dieckmann 1980).

4. ***Holospora obtusa*** (ex Hafkine 1890) Gromov and Ossipov 1981, 351^{VP}
ob.tu'sa. L. fem. adj. *obtusa* obtuse, blunt.

Short, fusiform, reproductive rod about 3.0 μm long that undergoes binary fission and grows in infective form up to 20 μm long. Ends of rod rounded (Fig. BXII.α.58). Found in *P. caudatum* in the macronucleus. Diagnostic characteristics are given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

*Editorial Note: This genus was described by J. R. Preer, Jr. and L. B. Preer (1984) in the first edition of *Bergey's Manual of Systematic Bacteriology*. We have used their descriptions and figures and have added newer information.

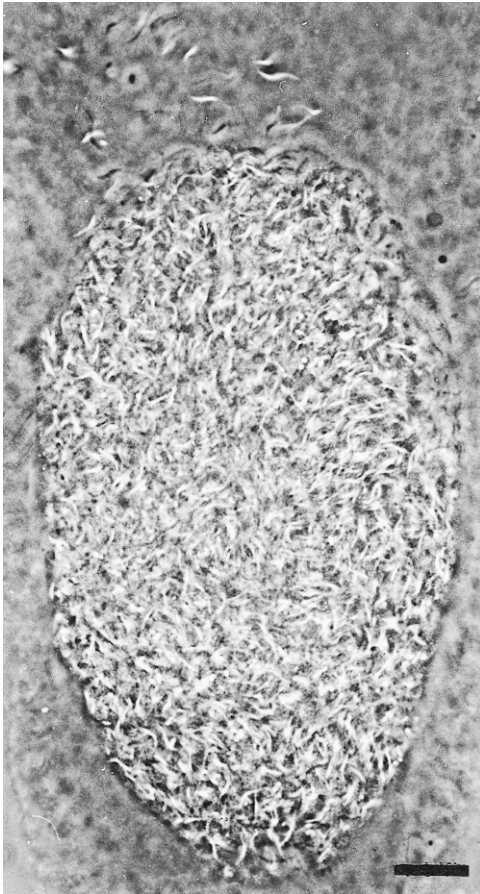


FIGURE BXII.α.56. Macronucleus of *Paramecium biaurelia* stock 562. The spiral endosymbiont filling the macronucleus is *Holospora caryophila*. Osmium–lacto–orcein preparation, whole mount, bright phase-contrast. Bar = 10 μm. (Reproduced with permission from L.B. Preer, *Journal of Protozoology* 16: 570–578, 1969, ©Society of Protozoologists.)

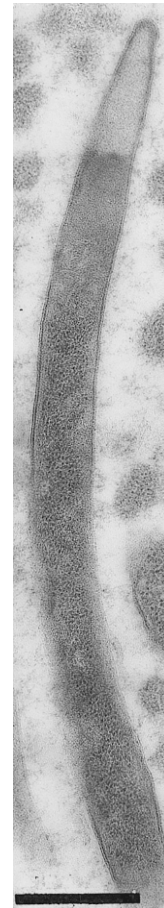


FIGURE BXII.α.57. *Holospora caryophila* of *Paramecium biaurelia* stock 562, spiral form. Longitudinal section. Bar = 0.5 μm. (Reproduced with permission from L.B. Preer, *Journal of Protozoology* 16: 570–578, 1969, ©Society of Protozoologists.)

Type strain: The original description and illustration was given by Hafkine (1890).

Additional Remarks: Clone M.115 of *P. caudatum* containing *H. obtusa* in its macronuclei has been deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University (Gromov and Ossipov, 1981).

5. “*Holospora acuminata*” Ossipov, Skoblo, Borchsenius, Rautian and Podlipaev 1980, 927.

Lives in the micronucleus of *Paramecium bursaria* (Ossipov et al., 1980). Reproductive form short fusiform rod; infectious form 5.0–8.0 × 0.5–0.6 μm, straight, both ends

tapered. The species name has not been validly published. There is, however, little doubt that it belongs to the genus *Holospora*, because of many biological features shared with the three species *H. undulata*, *H. obtusa*, and *H. elegans*. In addition, they are recognized with an oligonucleotide probe designed for *H. obtusa*.

The mol% G + C of the DNA is: not determined.

Deposited strain: AC61-10.

Additional Remarks: In stock AC61-10 of *P. bursaria* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, State University of St. Petersburg, Russia).

Other Organisms

1. “*Holospora bacillata*” Fokin and Sabaneyeva 1993, 393.

Lives in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva, 1993) and *Paramecium woodruffi* (Fokin et al., 1996; Fokin and Sabaneyeva, 1997). Infectious form 5–

17.0 × 0.7–0.8 μm, straight, both ends rounded. Diagnostic characteristics as given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

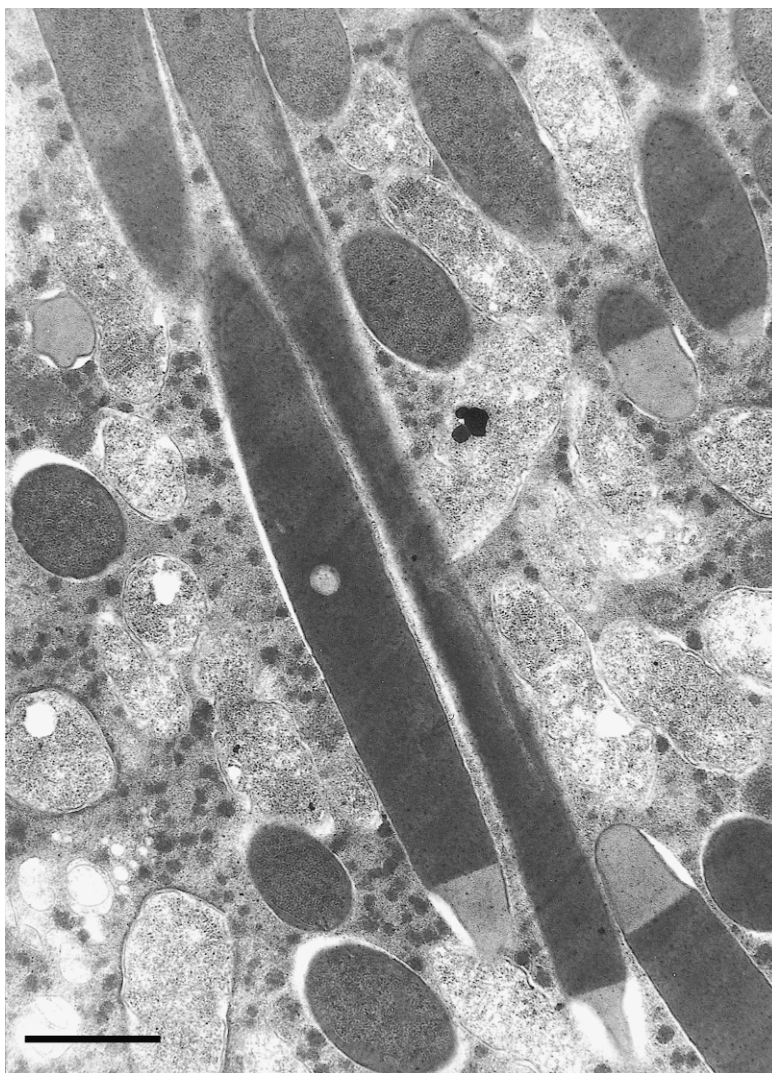


FIGURE BXII.α.58. *Holospora obtusa*, macronuclear symbiont of *Paramecium caudatum*. Infectious form stains darkly; the noninfectious form is light. Bar = 4.3 μm (Reproduced with permission from H.-D. Görtz, 1980. In Schwemmler and Schenk (Editors), *Endocytobiology: Endosymbiosis and Cell Biology*, Vol. 1, ©Walter deGruyter and Co, pp. 381–392.)

2. “*Holospora curvata*” Fokin and Sabaneyeva 1993, 393.

Lives in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva, 1993). Infectious form 12–20 × 0.7–0.9 μm, curved, both ends rounded. Diagnostic characteristics as given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

3. “*Holospora curviuscula*” Borchsenius, Skoblo and Ossipov 1983, 96.

Lives in the macronucleus (sometimes also micronucleus) of *P. bursaria*. Reproductive form short, spindle shaped; infectious form 4.0–10.0 × 0.4–0.5 μm, slightly curved rod with tapered ends. Diagnostic characteristics as given in Table BXII.α.45.

The mol% G + C of the DNA is: not determined.

4. “*Holospora recta*” Fokin 1991, 139.

rec.ta. L. adj. *rectus* straight; M.L. fem. adj. *recta* straight.

Lives in the micronucleus of *Paramecium caudatum* (Fokin, 1991). Infectious form 10–15 × 0.7–1.0 μm, straight, one end rounded, one end tapered. Diagnostic characteristics as given in Table BXII.α.45. Whereas all other species listed in this chapter appear to be taxonomically sound, this has been questioned for “*H. recta*” by Rautian and Ossipov (personal communication), who found a strain of *H. elegans* with a number of individuals exhibiting “*H. recta*”-like features. The question may finally be resolved after molecular data have been obtained from the different strains.

The mol% G + C of the DNA is: not determined.

Genus Incertae Sedis II. Caedibacter (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP*}

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Cae' di. bac. ter. L. n. *caedes* act of killing; M.L. masc. n. *bacter* the masculine equivalent of the Gr. neut. n. *bactrum* a rod; masc. n. *caedibacter* the bacterium which kills.

Straight rods or coccobacilli $0.4\text{--}1.0 \times 1.0\text{--}4.0\text{ }\mu\text{m}$. Gram negative, nonflagellated, nonmotile. Intracellular (endosymbiotic) bacteria in *Paramecium*. Culture free of *Paramecium* impossible or exceedingly difficult. The ability to produce refractile inclusion (R) bodies was seen as a unique feature of the genus. R bodies are proteinaceous ribbons, approximately $10\text{ }\mu\text{m}$ long, coiled inside the bacterial cell (Figs. BXII.α.59, BXII.α.60, and BXII.α.61). The hollow cylindrical structure formed may be $0.4\text{--}0.8\text{ }\mu\text{m}$ long and about $0.4\text{ }\mu\text{m}$ in diameter. R bodies unroll when ingested into a phagosome and under certain *in vitro* conditions (for details see Preer et al., 1974; Quackenbush, 1988; Pond et al., 1989). R bodies are associated with spherical phage-like structures or covalently closed circular DNA plasmids. All species are toxic to certain sensitive strains of paramecia; bacteria confer

killer-trait or mate killer-trait upon their host cells. An exception is "*C. macronucleorum*" from *Paramecium duboscqui*, where no toxic effects were observed. Cells containing R bodies are usually larger than cells that do not contain R bodies, and contain many spherical phage-like structures of covalently closed, circular, DNA plasmids. Recently, a symbiotic bacterium from *Acanthamoeba* was included in the genus *Caedibacter* because of its sequence similarity to *Caedibacter caryophilus* (Horn et al., 1999). This new *Caedibacter* species, *Candidatus Caedibacter acanthamoebae*, does not establish R bodies. Also interesting is the relatively close relationship of *Caedibacter* to the genus *Holospira* and to the "NHP bacterium", etiologic agent of necrotizing hepatopancreatitis in shrimp (Loy et al., 1996; Horn et al., 1999).

The mol% G + C of the DNA is: 34–44.

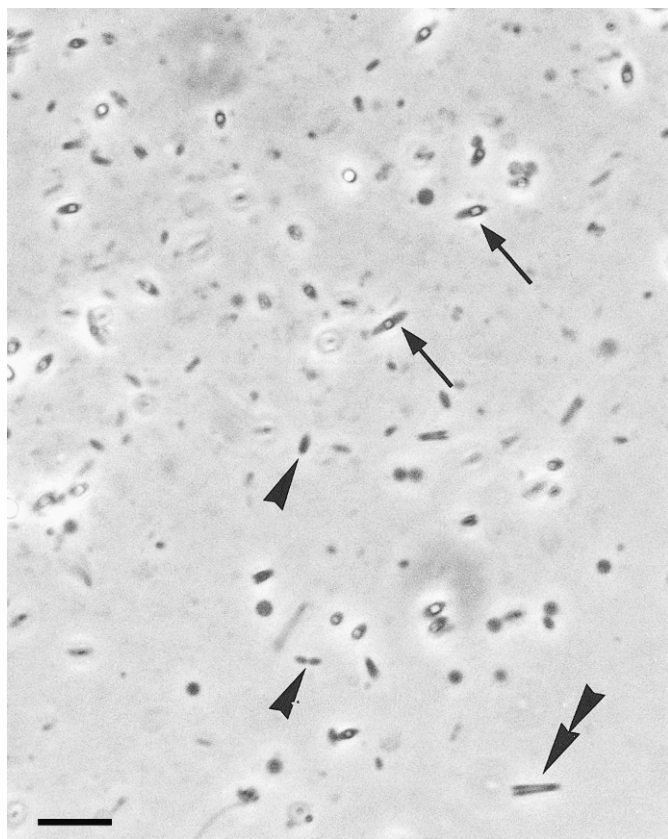


FIGURE BXII.α.59. Fresh squash of *Paramecium novaurelia*, washed free of bacteria, viewed by bright phase-contrast. Note *Caedibacter caryophilus* present in two forms: small rods (arrowheads) and large spindle-shaped cells containing refractile bodies (arrows) characteristic of the genus. Free R bodies, partly stretched, (double arrowhead) are also visible. Bar = $2.4\text{ }\mu\text{m}$.

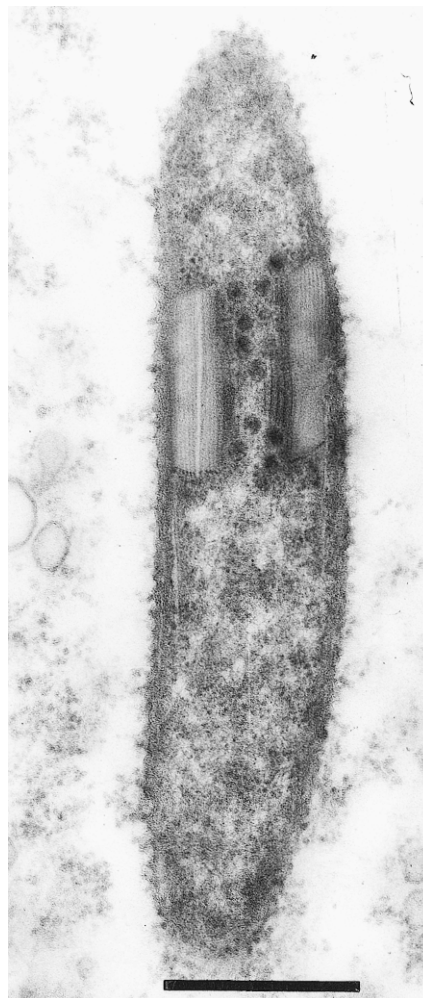


FIGURE BXII.α.60. *Caedibacter varicaedens*, endosymbiont of *Paramecium biaurelia* stock 7. Note spherical phages inside the coiled R body. Longitudinal section. Bar = $0.5\text{ }\mu\text{m}$. (Reproduced with permission from J.R. Preer, Jr. and A. Jurand, *Genetical Research* 12: 331–340, 1968, ©Cambridge University Press.)

*Editorial Note: This genus was described by J.R. Preer, Jr. and L.B. Preer (1984) in the first edition of *Bergey's Manual of Systematic Bacteriology*. The authors have used their descriptions and added new information.

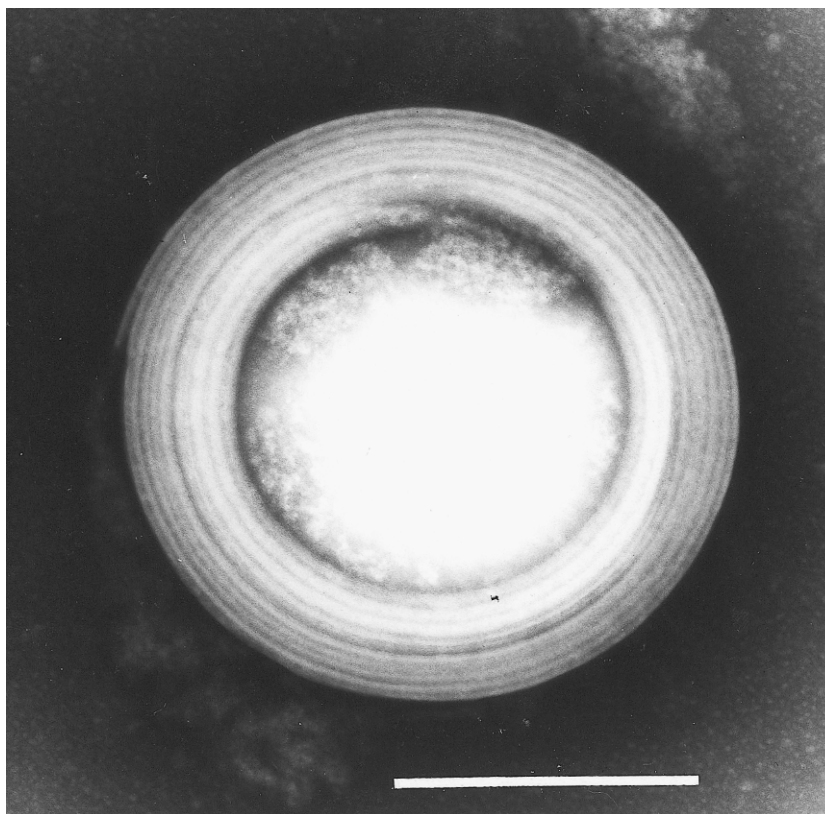


FIGURE BXII.α.61. Intact R body from *Caedibacter varicaedens* of *Paramecium biaurelia* stock 511. Phosphotungstic acid. Bar = 2.5 μ m. (Reproduced with permission from J.R. Preer, Jr. et al., Bacteriological Reviews 38: 113–163, 1974, ©American Society for Microbiology.)

Type species: ***Caedibacter taeniospiralis*** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140 (*Caedobacter taeniospiralis* [sic] Preer, Preer and Jurand 1974, 157.)

FURTHER DESCRIPTIVE INFORMATION

The distinguishing characteristic of *Caedibacter* species is the presence of refractile (R) bodies. Between less than 10% and up to 50% of the bacteria in a host cell contain R bodies. Cells containing R bodies are called “brights”; cells without R bodies are called “non-brights”. Only a very weak infectivity, if any, is observed for non-brights. The coiled R body, a distinctive structure, is seen with bright phase-contrast microscopy either as doughnut-shaped or as a pair of parallel rods, depending upon its orientation (Fig. BXII.α.62). Although the structure is readily resolved in bright phase-contrast, it is often obscure in dark phase-contrast, because dark phase-contrast optics are usually of such high contrast that phase reversal occurs in most aqueous media. R bodies themselves appear to result from the induction of phage-like or plasmid-like extrachromosomal DNAs (Quackenbush, 1988; Pond et al., 1989). The induction appears to be lethal: R body-containing cells do not have the capacity to reproduce. It is interesting that R bodies have been observed in free-living bacteria belonging to the genus *Pseudomonas* (Lalucat et al., 1979). The toxicity of *C. taeniospiralis* and *C. pseudomutans*, formerly known as kappa, is produced by the ingestion of R body-containing kappas by sensitive strains of paramecia.

R bodies can be induced to unroll (Fig. BXII.α.63) and in some cases, reroll; they have been shown to unroll in the food

vacuoles of sensitive paramecia. In some species of *Caedibacter*, the R body unrolls from the inside (Fig. BXII.α.64); in others, from the outside. Rupture of the membrane of the food vacuole occurs, and the contents of the food vacuole, including the R bodies, pass into the cytoplasm of the paramecium. The toxins themselves have never been obtained in soluble form and their nature is unknown. Ingestion of the fourth species, *C. paraconjugatus*, does not produce toxic effects on sensitives. Instead it is a mate-killer, and sensitive paramecia die only after contact with paramecia bearing *C. paraconjugatus* during conjugation. The endosymbiont discovered by Estève (1978) in the macronucleus of a strain of *Paramecium caudatum* was later found again and described as *C. caryophilus* (Schmidt et al., 1987a; Euzéby, 1997).

Note added in proof: According to an analysis of 16S rRNA sequences, *C. taeniospiralis* (isolated from stock 51k of *Paramecium tetraurelia*) belongs to the *Gammaproteobacteria*, whereas *C. caryophilus* belongs to the *Alphaproteobacteria* (Beier et al., 2002).

FURTHER READING

- Heckmann, K. and H.D. Görtz. 1991. Procaryotic symbionts of ciliates. In Balows, Trüper, Dworkin, Harder and Schleifer (Editors), The Prokaryotes a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, Springer Verlag, Berlin, Heidelberg, New York. pp. 3865–3890.
- Pond, F.R., I. Gibson, J. Lalucat and R.L. Quackenbush. 1989. R-body producing bacteria. Microbiol. Rev. 53: 25–67.
- Preer, J.R., Jr., L.B. Preer and A. Jurand. 1974. Kappa and other endosymbionts in *Paramecium aurelia*. Bacteriol. Rev. 38: 113–163.

Quackenbush, R.L. 1988. Endosymbionts of killer paramecia. In Görtz (Editor), *Paramecium*, Springer Verlag, Heidelberg, New York. pp. 406–418.

Soldo, A.T. 1974. Intracellular particles in *Paramecium aurelia*. In Wagtendonk, v. (Editor), *Paramecium: A Current Survey*, Elsevier, Amsterdam. pp. 375–442.

List of species of the genus Caedibacter

1. ***Caedibacter taeniospiralis*** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP}
taen.i.o.spi.ra'l is. L. n. *taenia* ribbon; L. adj. *spiralis* coiled; M.L. masc. adj. *taeniospiralis* coiled ribbon.

Description of the species as for the genus. Rods, $0.4\text{--}0.7 \times 1.0\text{--}2.5 \mu\text{m}$. Found exclusively in the cytoplasm of *Paramecium tetraurelia*. Contain plasmids (Dilts, 1976). R bodies unroll from the inside and contain plasmids. Ingestion of R body-containing symbionts by sensitive paramecia causes development of small blisters or humps on their surface within 2–3 hours preceding the death of the paramecium.

The mol% G + C of the DNA is: 41 (Bd).

Type strain: ATCC 30632.

Additional Remarks: The type strain of *Caedibacter taeniospiralis* was isolated from stock 51 of *Paramecium tetraurelia* (ATCC 30632) (Preer et al., 1974)

2. ***Caedibacter caryophilus*** Schmidt, Görtz and Quackenbush 1987a, 461^{VP}
ca.ry.o'phi.lus. Gr. n. *caryum* nucleus; Gr. adj. *philus* loving; M.L. adj. *caryophilus* nucleus loving.

In macronucleus of *P. caudatum*. Cells without R bodies $1\text{--}1.5 \times 0.4 \mu\text{m}$, cells with R bodies $1.5\text{--}2.5 \times 0.7 \mu\text{m}$. R bodies unrolling from the inside. Outer terminus of unrolled R bodies blunt, inner terminus acute. R bodies associated with phages. Width of R bodies $0.8 \mu\text{m}$. Sensitive strains of *Paramecium* are killed by paralysis. Co-infections with *Holospira* species in natural populations.

The mol% G + C of the DNA is: 35 (T_m).

Type strain: ATCC 50168.

GenBank accession number (16S rRNA): X71837.

Additional Remarks: The type strain of *Caedibacter caryophilus* was isolated from stock C221 of *P. caudatum* (ATCC 50168) (Schmidt et al., 1987a).

3. ***Caedibacter paraconjugatus*** Quackenbush 1982, 266^{VP} (Effective publication: Quackenbush 1978, 186.)
para.con.ju.ga'tus. Gr. prep. *para* alike; L. part. adj. *conjugatus* conjugated; also the specific epithet of a mate killer (*Pseudocaedibacter conjugatus*); M.L. masc. part. adj. *paraconjugatus* similar to mate killers.

Small rods. Fewer than 1% of the cells contain R bodies, which are smaller than those found in the other species of

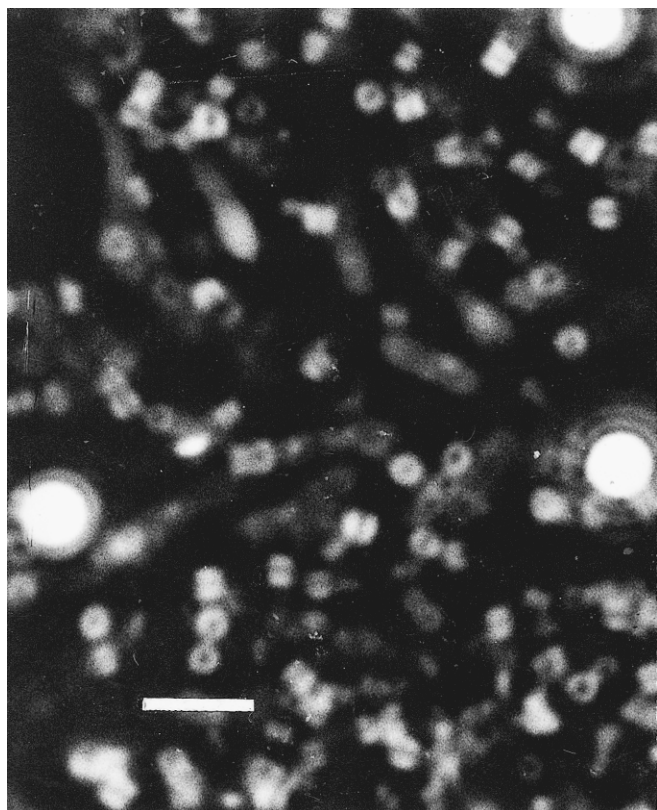


FIGURE BXII.α.62. Isolated R bodies from *Caedibacter varicaedens* of *Paramecium biaurelia* stock 7, viewed with bright phase-contrast. R bodies appear doughnut-shaped when viewed on end, and as two parallel rods when viewed from the side. The large bright spheres are latex particles. Bar = 2 μm .

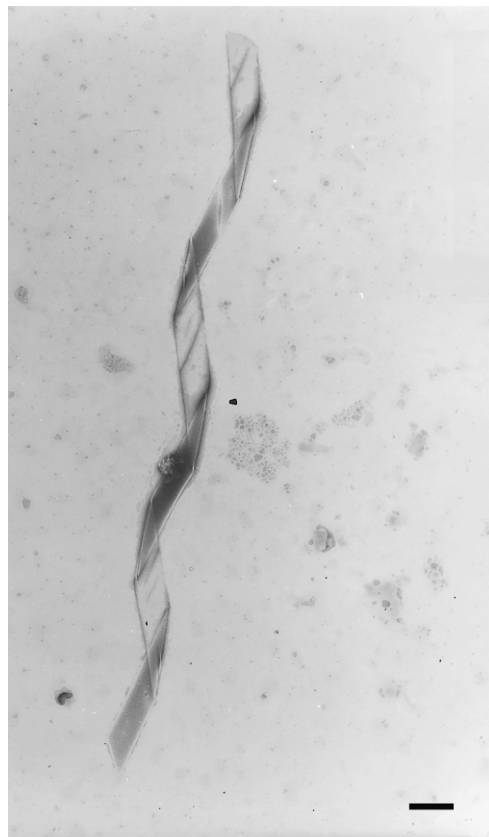


FIGURE BXII.α.63. Unrolled R body isolated from *Caedibacter varicaedens* of *Paramecium biaurelia* stock 1039. Phosphotungstic acid. Bar = 1 μm . (Reproduced with permission from J.R. Preer Jr. et al., Bacteriological Reviews 38: 113–163, 1974, ©American Society for Microbiology.)

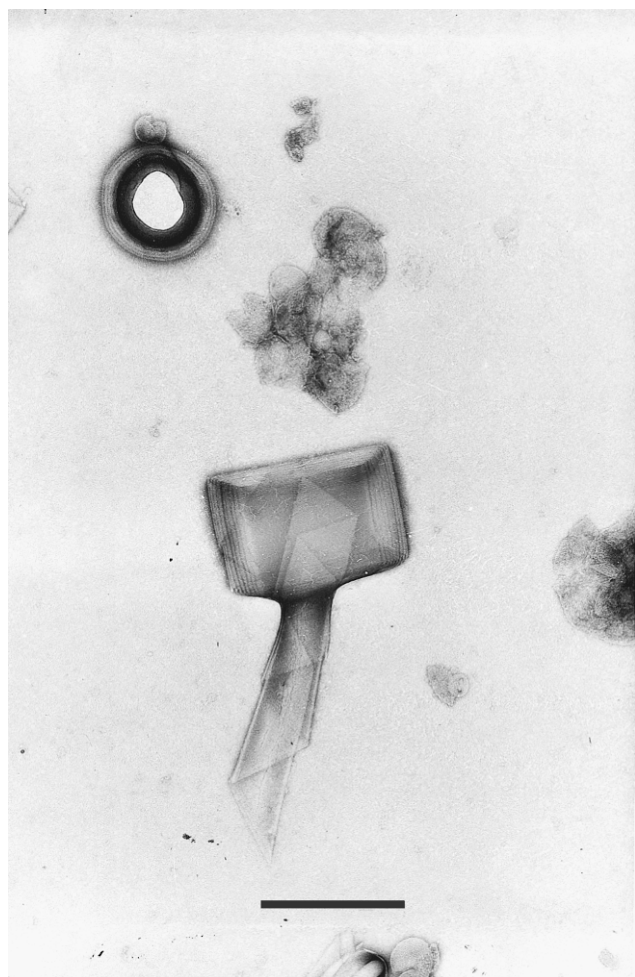


FIGURE BXII.α.64. R bodies isolated from *Caedibacter taeniospiralis* of *Paramecium tetraurelia* stock 51. Note intact doughnut-shaped R body and, below, an R body unrolling from inside. Phosphotungstic acid. Bar = 0.5 μm . (Reproduced with permission from L.B. Preer, *Journal of Cell Science*, 11: 581–600, 1972, ©Company of Biologists, Ltd.)

Caedibacter. Ingestion of cells by sensitive paramecia does not cause any observable effects. Cell-to-cell contact between host and sensitive paramecia is required for toxic effects (mate killing) to be observed in the sensitive paramecia. Found in *P. biaurelia*. Contain phage-like structures.

The mol% G + C of the DNA is: not determined.

Type strain: ATCC 30638.

Additional Remarks: The type strain of *Caedibacter parajugatus* was isolated from stock 570 of *Paramecium biaurelia* (ATCC 30638) (Preer et al., 1974).

4. ***Caedibacter pseudomutans*** Quackenbush 1982, 266^{VP} (Effective publication: Quackenbush 1978, 186.)
pseu.do.mu'tans. Gr. adj. *pseudo* false; L. part. adj. *mutans* changing; M.L. part. adj. *pseudomutans* false changing, referring to the fact that it was once thought to be a mutant of *C. taeniospiralis* (Dippell, 1950).

Cigar-shaped rods approximately $0.5 \times 1.5 \mu\text{m}$. Found in *P. tetraurelia*.

The mol% G + C of the DNA is: 44 (Bd).

Type strain: ATCC 30633.

Additional Remarks: The type strain of *Caedibacter pseudomutans* was isolated from stock 51ml of *Paramecium tetraurelia* (ATCC 30633) (Preer et al., 1974).

5. ***Caedibacter varicaedens*** Quackenbush 1982, 266^{VP} (Effective publication: Quackenbush 1978, 186.)
vari.cae'dens. L. adj. *varis* different; L. v. *caedo* to kill; M.L. part. adj. *varicaedens* killing in different ways.

Rods $0.4\text{--}1.9 \times 2\text{--}4 \mu\text{m}$. Different strains cause either vacuolization or paralysis or rapid reverse rotation while swimming (spin killing) in sensitive paramecia. R bodies unroll from the outside. One of the commonest killers of *P. biaurelia*. Most strains contain spherical phage-like structures (Fig. BXII.α.60).

The mol% G + C of the DNA is: 40–41 (Bd).

Type strain: ATCC 30637.

Additional Remarks: The type strain of *Caedibacter varicaedens* was isolated from stock 7 of *Paramecium biaurelia* (ATCC 30637) (Preer et al., 1974).

6. ***Candidatus Caedibacter acanthamoebae*** Horn, Fritsche, Gautom, Schleifer and Wagner 1999, 364.
a'canth.a.moe.bae. L. gen. sing. n. *acanthamoebae* of *Acanthamoeba*, genus name of host protozoa.

Phylogenetic position, *Alphaproteobacteria*; not cultivated on cell-free media; Gram reaction, negative; rod-shaped $\sim 0.7\text{--}3.3 \mu\text{m}$ in length, $0.22\text{--}0.33 \mu\text{m}$ in diameter; basis of assignment, 16S rDNA sequence (accession number AF1321138) and nucleotide S-S-CaeAc-998-a-A-18 (5'-TCTTGTCTCCGCGATCCC-3'); association and host, intracellular symbiont of *Acanthamoeba polyphaga* HN-3; mesophilic (Horn et al., 1999).

Other Organisms

1. "*Caedibacter macronucleorum*" Fokin and Görtz 1993, 322.
ma.cro.nu.cle.o'rum. Gr. adj. *macro* big; L. n. *nucleus* nucleus; *macronucleorum* of the macronucleus.

In macronucleus of *Paramecium duboscqui*. Cells without R bodies $1\text{--}1.5 \times 0.4 \mu\text{m}$, cells with R bodies $1.5\text{--}2.5 \times 0.7 \mu\text{m}$. R bodies unrolling from the inside. Outer terminus of unrolled R bodies blunt, inner terminus acute. R bodies associated with phages. Width of R bodies $0.8 \mu\text{m}$. Sensitive strains of *Paramecium* are killed by paralysis. Co-infections with *Holospira* species in natural populations. As was the

rule for intracellular bacteria in protozoa before the advent of molecular sequencing, this organism was named according to morphology and biological behavior. "*Caedibacter macronucleorum*" had been grouped into the genus *Caedibacter*, because it was found as an intracellular bacterium in a protozoon and because of its ability to form R bodies like the valid species of the genus. Since no molecular data have been obtained to date, the true phylogenetic position of the bacterium is not known.

Genus Incertae Sedis III. Lyticum (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 141^{VP*}

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Ly'ti.cum. L. adj. *lyticus* dissolving; M.L. neut. n. *Lyticum* dissolver.

Large rods 0.6–0.8 μm in diameter, straight, curved, or spiral. Length of single forms 3.0–5.0 μm . Numerous **peritrichous flagella**. Although cultivation independently from *Paramecium* has been reported, it has not been confirmed. Produce labile **toxins which kill** sensitive strains of paramecia **very quickly by lysis**. Gram negative. Nonmotile or almost so, in spite of numerous, well-developed flagella (Fig. BXII.α.65). Occurs in *Paramecium biaurelia*, *P. tetraurelia*, and *P. octaurelia* (Fig. BXII.α.66).

The mol% G + C of the DNA is: 27–49.

Type species: ***Lyticum flagellatum*** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140.

FURTHER DESCRIPTIVE INFORMATION

These very large endosymbionts are unique due to their conspicuous flagella and rapidly acting toxins (less than 30 minutes at room temperature). The nature of the toxin has not been revealed. *Paramecium triaurelia*, *P. pentaurelia*, and *P. novaurelia* are particularly sensitive to the toxins. The substantially different

values for mol% G + C of DNA reported by Behme and by Soldo (see Preer et al., 1974 for a discussion) are unresolved.

DIFFERENTIATION OF THE GENUS *LYTICUM* FROM OTHER GENERA

Lyticum flagellatum is a straight rod found in *Paramecium tetraurelia* and *P. octaurelia*, while *L. sinuosum* is a strikingly curved rod found in *Paramecium biaurelia*.

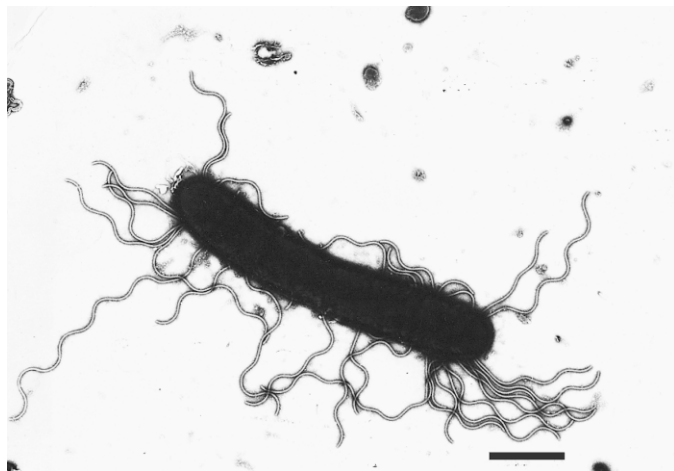


FIGURE BXII.α.65. *Lyticum flagellatum* isolated from *Paramecium octaurelia* stock 327. Phosphotungstic acid. Bar = 1 μm . (Reproduced with permission J. R. Preer, Jr. et al., Bacteriological Reviews 38: 113–163, 1974, ©American Society for Microbiology.)



FIGURE BXII.α.66. *Paramecium tetraurelia* stock 239 bearing endosymbiont *Lyticum flagellatum*. The numerous black rods throughout the cytoplasm are the endosymbionts. Osmium–lacto–orcein preparation, whole mount, dark phase-contrast. Bar = 20 μm . (Reproduced with permission J.R. Preer, Jr. et al., Bacteriological Reviews 38: 113–163, 1974, ©American Society for Microbiology.)

List of species of the genus *Lyticum*

1. ***Lyticum flagellatum*** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP}
fla.gel.la'tum. L. neut. n. part. adj. *flagellatum* flagellated.

Straight rods 0.6–0.8 \times 2.0–4.0 μm , with many peritri-

chous flagella (Fig. BXII.α.65). Originally called lambda. Found within the cytoplasm of *Paramecium tetraurelia* and *P. octaurelia*. Stock 299 of *P. octaurelia* containing lambda does not require folic acid, whereas symbiont-free lines of 299 do (Soldo and Godoy, 1973).

The mol% G + C of the DNA is: 27 (Bd) in one strain (Soldo) and 49 (Bd) in another (Behme; Preer et al. 1974).

Type strain: 299, ATCC 30700.

*Editorial Note: This genus was described by J.R. Preer, Jr. and L.B. Preer (1984) in the first edition of *Bergey's Manual of Systematic Bacteriology*. The authors have used their descriptions and figures and have added newer information.

2. **Lyticum sinuosum** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP}
sin' u.o.sum. L. neut. adj. *sinuosum* winding, sinuous.
 Curved or spiral rods $0.7\text{--}0.9 \times 2.0\text{--}10.0 \mu\text{m}$, sometimes

forming chains of 2–3 cells. Originally called sigma. Found within the cytoplasm of *Paramecium biaurelia*.
The mol% G + C of the DNA is: 45 (Bd).
Type strain: 114, ATCC 30696.

Genus Incertae Sedis IV. Candidatus Odysella Birtles, Rowbotham, Michel, Pitcher, Lascola, Alexou-Daniel and Raoult 2000, 71

THE EDITORIAL BOARD

Od.ys.sel' la. Gr. dim. fem. n. *Odysella* pertaining to Odysseus.

Motile, rod-shaped ($0.2\text{--}0.5 \times 0.7\text{--}1.0 \mu\text{m}$) **intracytoplasmic parasite** of *Acanthamoeba* spp. Does not grow axenically. Most virulent at temperatures above 30°C.

The mol% G + C of the DNA is: 41 ± 1 (T_m).

Type species: *Candidatus Odysella thessalonicensis* Birtles, Rowbotham, Michel, Pitcher, Lascola, Alexou-Daniel and Raoult 2000, 71.

FURTHER DESCRIPTIVE INFORMATION

Electron micrographs show that the organisms are present in the cytoplasm of infected *Acanthamoeba* cells and that at lower growth temperatures the bacterial cells are sometimes surrounded by a clear zone containing vesicles that appear to have budded from the bacterial cell membrane.

The organism infects the following *Acanthamoeba* species: *A. polyphaga*, *A. lenticulata*, *A. castellanii*, *A. quinaludunensis*, and *A. comandoni*.

Comparisons of 16S rDNA sequences place the organism in the *Alphaproteobacteria*; however, its 16S rDNA sequence was not closely related to those of any of the bacteria to which it was compared. It was most similar to that of two other intracellular parasites: *Holospora obtusa* and *Caedibacter caryophilus*.

ENRICHMENT AND ISOLATION PROCEDURES

Candidatus Odysella thessalonicensis was first isolated from an *Acanthamoeba* sp. obtained from an air-conditioning system. It was propagated on *A. polyphaga* Linc Ap-1 using the methods of Birtles et al. (1996).

MAINTENANCE PROCEDURES

Suspensions of infected *Acanthamoeba* cells can be stored at -70°C (Birtles et al., 1996).

List of species of the genus Odysella

1. *Candidatus Odysella thessalonicensis* *sp. nov.* Birtles, Rowbotham, Michel, Pitcher, Lascola, Alexou-Daniel and Raoult 2000, 71.
thess.al.lon'i.cen'sis. M.L. masc. n. adj. *thessalonicensis* pertaining to the Greek city Thessalonika, where the organism was isolated.

The genus description and the species description are identical.

The mol% G + C of the DNA is: 41 ± 1 (T_m).

Type strain: L13 (only described strain).

GenBank accession number (16S rRNA): AF069496.

Genus Incertae Sedis V. Candidatus Paracaedibacter Horn, Fritsche, Gautom, Schleifer and Wagner 1999, 364

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

For a description see the list of species.

TAXONOMIC COMMENTS

A formal description and diagnosis has not yet been published. Because of the relatively low overall 16S rDNA sequence simi-

larities (87–88%) of the two new species *Candidatus Paracaedibacter acanthamoebae* and *Candidatus Paracaedibacter symbiosus*, Horn et al. (1999) propose the provisional classification of these symbionts in the new genus *Candidatus Paracaedibacter*.

List of species of the genus Candidatus Paracaedibacter

1. *Candidatus Paracaedibacter acanthamoebae* Horn, Fritsche, Gautom, Schleifer and Wagner 1999, 364.
 Not cultivated on cell-free media; Gram negative; rod shaped, $\sim 1.3\text{--}1.7 \times 0.22\text{--}0.24 \mu\text{m}$. Based on 16S rDNA sequence and nucleotide probe S-S-PcaeC9-217-a-A-18 (5'-

GGGCTGCTCAATTGGCGA-3'), *Candidatus Paracaedibacter acanthamoebae* is assigned to the *Alphaproteobacteria*. Intracellular symbiont of *Acanthamoeba* sp. UWC9. Mesophilic.
GenBank accession number (16S rRNA): AF132137.

2. *Candidatus* Paracaedibacter symbiosus Horn, Fritsche, Gautom, Schleifer and Wagner 1999, 364.

Not cultivated on cell-free media; Gram negative; rod shaped, $\sim 1.3\text{--}1.7 \times 0.22\text{--}0.24 \mu\text{m}$. Based on 16S rDNA sequence and nucleotide probe S-S-PcaeE39-217-a-A-18 (5'-GGGCTGTTCCCTTTAGCGA-3'), *Candidatus* Paracaedibac-

ter symbiosus is assigned to the *Alphaproteobacteria*. Intracellular symbiont of *Acanthamoeba* sp. UWE39. Mesophilic. GenBank accession number (16S rRNA): AF132139.

Genus Incertae Sedis VI. **Pseudocaedibacter** Quackenbush 1982, 267^{VP} (Effective publication Quackenbush 1978, 186)*

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Pseu.do.cae'di.bac.ter. Gr. adj. *pseudo* false; M.L. n. *Caedibacter* genus of endosymbionts that include organisms commonly known as kappa; M.L. masc. n. *Pseudocaedibacter* false kappa particles.

Rods $0.25\text{--}0.7 \times 0.5\text{--}4 \mu\text{m}$. Gram negative. Nonmotile. **Do not produce R-body-containing cells.** May or may not confer a killer trait upon their host paramecia. Occurs in *Paramecium primaurelia*, *P. biaurelia*, *P. pentaurelia*, and *P. octaurelia*. Whether lacking the ability to produce R bodies justifies separation of the genus *Pseudocaedibacter* from the genus *Caedibacter* may be questioned because the newly found endosymbiont from *Acanthamoeba polyphaga*, *Candidatus* Caedibacter acanthamoebae, does not establish R bodies but showed 93% sequence similarity (16S rRNA) to *Caedibacter caryophilus* and was therefore included in the genus *Caedibacter* (Horn et al., 1999).

The mol% G + C of the DNA is: 35–39.

Type species: ***Pseudocaedibacter conjugatus*** (ex Preer, Preer and Jurand 1974) Quackenbush 1982, 267 (Effective publication: Quackenbush 1978, 187) (*Caedibacter conjugatus* Preer, Preer and Jurand 1974, 157.)

FURTHER DESCRIPTIVE INFORMATION

Pseudocaedibacter is much like *Caedibacter*, except that no R bodies are found. It includes the typical mate-killing forms (*P. conjugatus*, called mu), the small killing forms of *Paramecium octaurelia* (*Pseudocaedibacter minuta*, gamma), and the nondescript forms with no killing action (*P. falsus*, nu).

TAXONOMIC COMMENTS

For formal reasons, "*Pseudocaedibacter glomeratus*" is listed under Other Organisms, because the species name has not been validated. However, it should be stated that the validated species, too, were only included in the genus because of a few biological features. As no molecular or physiological data are available for any of the species, the whole genus and its species appear arbitrary.

*Editorial Note: This genus was described by J.R. Preer, Jr. and L.B. Preer (1984) in the first edition of *Bergey's Manual of Systematic Bacteriology*. The authors have used their descriptions and figures and have added newer information.

List of species of the genus *Pseudocaedibacter*

1. ***Pseudocaedibacter conjugatus*** (ex Preer, Preer and Jurand 1974) Quackenbush 1982, 267^{VP} (Effective publication: Quackenbush 1978, 187) (*Caedibacter conjugatus* Preer, Preer and Jurand 1974, 157.)
con.ju.ga'tus. L. masc. part. adj. *conjugatus* conjugated.

Rods $0.3\text{--}0.5 \times 1\text{--}4 \mu\text{m}$. The species is called mu and (except for *Caedibacter paraconjugatus*) is the only symbiont responsible for the mate killer phenotype in the *Paramecium aurelia* complex (Fig. BXII.α.67). It produces the toxin capable of killing sensitive strains of *Paramecium* only after

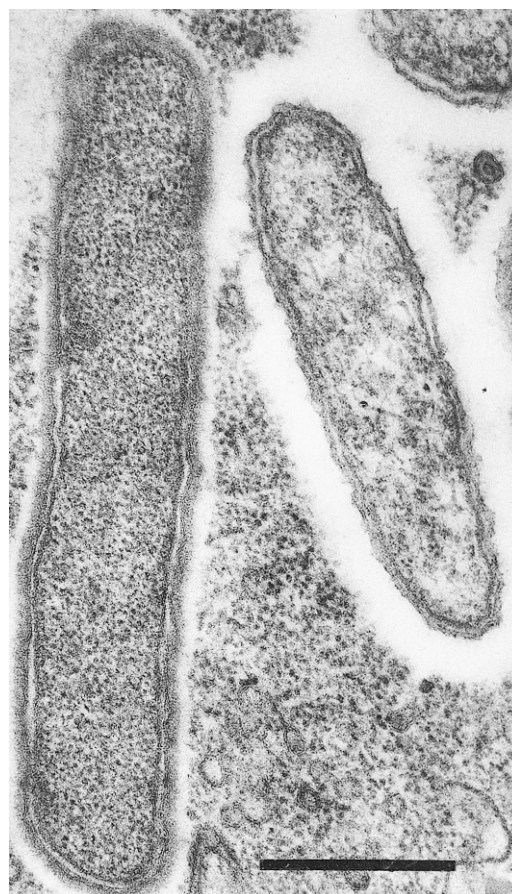


FIGURE BXII.α.67. Two endosymbionts present in a single cell of *Paramecium octaurelia* stock 131. Left, *Tectibacter vulgaris* (note electron-dense material surrounding outer membrane); right, *Pseudocaedibacter conjugatus*. Longitudinal section. Bar = $0.5 \mu\text{m}$. (Reproduced with permission from J.R. Preer, Jr. et al., *Bacteriological Reviews* 38: 113–163, 1974, ©American Society for Microbiology.)

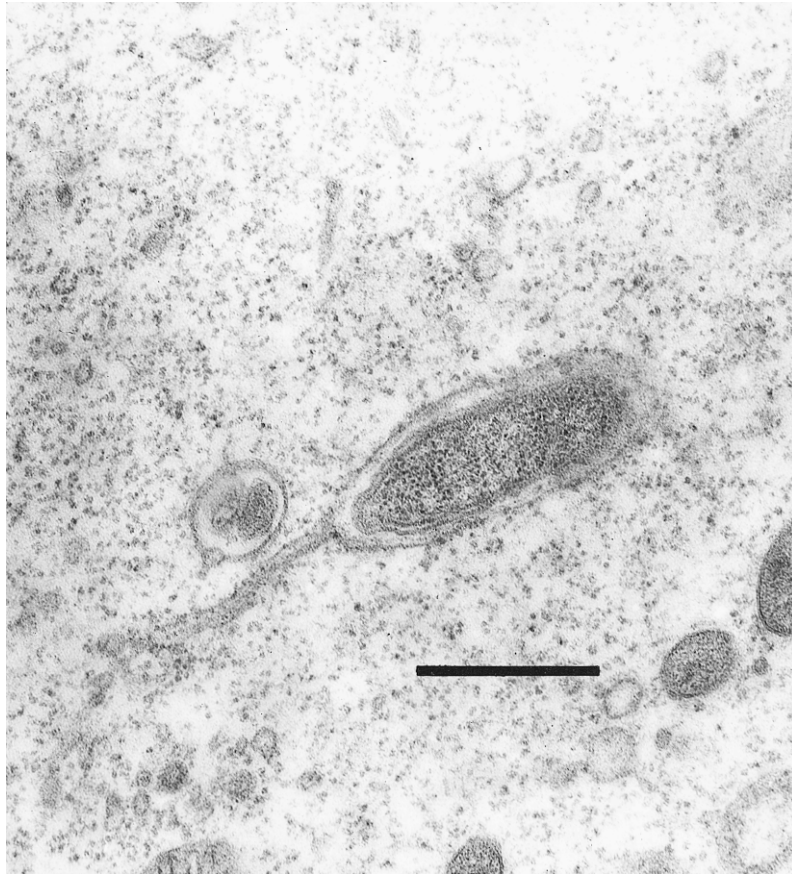


FIGURE BXII.α.68. *Pseudocaedibacter minutus*, endosymbiont of *Paramecium octaurelia* stock 565. No additional outer membrane. Longitudinal section. Bar = 0.5 μm. (Reproduced with permission from A. Jurand.)

cell-to-cell contact between killers and sensitives at conjugation. Cultivation outside the cytoplasm of *Paramecium* has been reported to occur on a very complex medium (Williams, 1971). Found in the cytoplasm of *Paramecium primaurelia* and *P. octaurelia*.

The mol% G + C of the DNA is: 35–37 (Bd).

Type strain: ATCC 30796.

Additional Remarks: The type strain of *P. conjugatus* is isolated from stock 540 of *Paramecium primaurelia* (ATCC 30796) (Preer et al., 1974).

2. ***Pseudocaedibacter minutus*** (Preer, Preer and Jurand 1974) Quackenbush 1982, 267^{VP} (Effective publication: Quackenbush 1978, 187) (*Caedibacter minutus* Preer, Preer and Jurand 1974, 157.)
mi.nu'tus. L. masc. adj. *minutus* small.

Rods, often double, 0.25–0.35 × 0.5–1.0 μm (singles). This very small cell is unique among the endosymbionts of *Paramecium* in being surrounded by an extra set of membranes, apparently continuous with the endoplasmic reticulum of its host (Fig. BXII.α.68). Although it never rises to high concentrations in the cytoplasm, the paramecia that bear it are nevertheless very strong killers. Found only in

the cytoplasm of *Paramecium octaurelia*. Originally called gamma.

The mol% G + C of the DNA is: 38 (Bd).

Type strain: ATCC 30699.

Additional Remarks: Isolated from stock 214 of *Paramecium octaurelia* (ATCC 30699) (Preer et al., 1974).

3. ***Pseudocaedibacter falsus*** (Preer, Preer and Jurand 1974) Quackenbush 1982, 267^{VP} (Effective publication: Quackenbush 1978, 187) (*Caedibacter falsus* Preer, Preer and Jurand 1974, 157.)
fal'sus. L. masc. adj. *falsus* false.

Rods 0.4–0.7 × 1.0–1.5 μm. No toxic action known, although the forms (called nu) found in *Paramecium pentau-relia* are said to increase the resistance of their hosts to the toxin produced by *Lyticum flagellatum* (Holtzman, 1959). The strains found in *Paramecium tetraurelia* were once regarded as mutants of *Caedibacter taeniospiralis* and called pi. Found also in the cytoplasm of *Paramecium biaurelia*.

The mol% G + C of the DNA is: 36 (Bd).

Type strain: ATCC 30640.

Additional Remarks: Isolated from stock 1010 of *P. biaurelia* (ATCC 30640) (Preer et al., 1974).

Other Organisms

1. "*Pseudocaedibacter glomeratus*" Fokin and Ossipov 1986, 1003.

Rods, $1.0\text{--}1.2 \times 0.3 \mu\text{m}$. No flagella. Gram negative, no central nucleoid. Bacteria are encircled by host membranes in a symbiontophoral vacuole. These vacuoles are surrounded by lacunae of endoplasmic reticulum with numerous ribosomes. No killing or toxic actions known.

The mol% G + C of the DNA is: not determined.

The type strain is in *Paramecium pentaurelia* strain Bp171, culture collection of the Laboratory of Protozoan Karyology of the Biological Research Institute of St. Petersburg State University, Russia.

Genus Incertae Sedis VII. "**Pseudolyticum**" Boss, Borchsenius and Ossipov 1987, 98*

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

pseu'do.ly.ti.cum. Gr. adj. *pseudo* false; L. adj. *lyticus* dissolving; M.L. neut. n. *Lyticum* genus of endosymbionts producing labile toxins which kill sensitive strains of paramecia very quickly by lysis; M.L. n. *Pseudolyticum* false *Lyticum*.

Straight rods, $2.0 \times 3.5\text{--}14.0 \mu\text{m}$. Bacteria are not infective and do not confer a killer trait on their host. Gram negative. Non-motile in spite of numerous flagella. Occur in *Paramecium caudatum*.

The mol% G + C of the DNA is: not determined.

Type species: "**Pseudolyticum multiflagellatum**" Boss, Borchsenius and Ossipov 1987, 98.

List of species of the genus *Pseudolyticum*

1. "**Pseudolyticum multiflagellatum**" Boss, Borchsenius and Ossipov 1987, 98.

mul.ti fla.gel.la'tum. L. neut. adj. *multus* many; L. neut. part. adj. *flagellatum* flagellated; *multiflagellatum* with many flagella.

As the only species, its characteristics are those of the genus. Straight rods $2.0 \times 3.5\text{--}14.0 \mu\text{m}$. Bacteria are not infective and do not confer a killer trait on their host. Occurs in *Paramecium caudatum*. Similar endosymbiotic bacteria in *P. caudatum* have been described by Dieckmann (1977). The bacteria described by Dieckmann were, however, infectious and motile when outside their host cells.

The mol% G + C of the DNA is: not determined.

Deposited strain: in stocks E39-3 and E39-10 (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, State University of St. Petersburg, Russia).

*Editorial Note: The genus has not been validly published, due to the custom of Russian protozoologists at the time of its description to publish new symbiont names in Russian journals. However named, species were only included in the genus because of a few biological features. As no molecular or physiological data are available for any of the species, the whole genus and its species appear arbitrary.

Genus Incertae Sedis VIII. **Tectibacter** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP}*

HANS-DIETER GÖRTZ AND HELMUT J. SCHMIDT

Tec.ti.bac'ter. L. masc. n. *tectum* covering; M.L. masc. n. *bacter* the equivalent of Gr. neut. n. *bactrum* a rod; M.L. masc. n. *Tectibacter* the bacterium with a covering.

Straight rods $0.4\text{--}0.7 \times 1\text{--}2 \mu\text{m}$. Distinguished by **outer covering around its cell wall**, visible in sections with the transmission electron microscope. Sparsely flagellated, peritrichous. No known

strains are toxic to protozoa. Gram negative. Often observed to be motile. Occur widely among strains of *Paramecium primaurelia*, *P. biaurelia*, *P. tetraurelia*, *P. sexaurelia*, and *P. octaurelia*, often with other symbionts (Fig. BXII.α.67).

*Editorial Note: This genus was described by J.R. Preer, Jr. and L.B. Preer (1984) in the first edition of *Bergey's Manual of Systematic Bacteriology*. We have used their descriptions and figures and have added newer information.

Type species: **Tectibacter vulgaris** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140.

List of species of the genus *Tectibacter*

1. **Tectibacter vulgaris** (ex Preer, Preer and Jurand 1974) Preer and Preer 1982, 140^{VP}

vulgar' is. L. masc. adj. *vulgaris* common.

The characteristics are as described for the genus.

The mol% G + C of the DNA is: not determined.

Type strain: ATCC 30697.

Additional Remarks: The type strain of *Tectibacter vulgaris* was isolated from stock 225 of *Paramecium sexaurelia* (ATCC 30697) (Preer et al., 1974).

Order III. **Rhodobacterales** *ord. nov.*

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Rho.do.bac.ter.a'les. M.L. masc. n. *Rhodobacter* type genus of the order; *-ales* suffix to denote order; M.L. fem. n. *Rhodobacterales* the *Rhodobacter* order.

The order *Rhodobacterales* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the order contains the family *Rhodobacteraceae*.

Description is the same as for the family *Rhodobacteraceae*.

Type genus: Rhodobacter Imhoff, Trüper and Pfennig 1984, 342.

Family I. **Rhodobacteraceae** *fam. nov.*

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Rho.do.bac.ter.a'ce.ae. M.L. masc. n. *Rhodobacter* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Rhodobacteraceae* the *Rhodobacter* family.

The family *Rhodobacteraceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Rhodobacter* (type genus), *Ahrensia*, *Albidovulum*, *Amaricoccus*, *Antarctobacter*, *Gemmobacter*, *Hirschia*, *Hyphomonas*, *Jannaschia*, *Ketogulonicigenium*, *Leisingera*, *Maricaulis*, *Methylarcula*, *Octadecabacter*, *Pannonibacter*, *Paracoccus*, *Pseudorhodobacter*, *Rhodobaca*, *Rhodothalassium*, *Rhodovulum*, *Roseibium*, *Roseinatronobacter*, *Roseivivax*, *Roseobacter*, *Roseovarius*, *Rubrimonas*, *Ruegeria*, *Sagittula*, *Staleyia*, *Stappia*, and *Sulfatobacter*. *Albidovulum*, *Leisingera*, *Pseudorhodobacter*, *Pannonibacter*, and *Jannaschia* were proposed after the cut-off date for inclusion in this volume (June 30, 2001) and are not described here (see Albuquerque et al.,

2002; Schaefer et al., 2002; Uchino et al., 2002; Borsodi et al., 2003, and Wagner-Döbler et al., 2003, respectively).

Family is phenotypically, metabolically, and ecologically diverse. Includes photoheterotrophs that can also grow photoautotrophically or chemotrophically under appropriate environmental conditions, chemoorganotrophs with either strictly aerobic or facultatively anaerobic respiratory metabolism, facultatively fermentative organisms, and facultative methylotrophs. Some denitrify. Many are aquatic. Many require NaCl for growth.

Type genus: Rhodobacter Imhoff, Trüper and Pfennig 1984, 342.

Genus I. Rhodobacter Imhoff, Trüper and Pfennig 1984, 342^{VP}

JOHANNES F. IMHOFF

Rho.do.bac' ter. Gr. n. *rhodon* the rose; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* a rod; M.L. masc. n. *Rhodobacter* red rod.

Cells are ovoid or rod-shaped, motile by polar flagella or non-motile, divide by binary fission or budding, may produce capsules and slime, and may form chains of cells. **Gram negative; belong to the Alphaproteobacteria**. Phototrophically grown cells **form vesicular or lamellar internal photosynthetic membranes**. The color of phototrophic cultures is yellow-green to yellow-brown, while aerobic cultures are pink to red. **Photosynthetic pigments are bacteriochlorophyll *a*** (esterified with phytol) and **carotenoids of the spheroidene series**. **Ubiquinone 10 is the major quinone**. **Major fatty acids are C₁₈ and C₁₆ saturated and monounsaturated fatty acids with C_{18:1} as predominant component**.

Photoheterotrophic growth occurs under anoxic conditions in the light with a variety of organic compounds as carbon and electron sources. **Photoautotrophic growth may be possible with hydrogen, sulfide, or thiosulfate as an electron donor**. **Chemotrophic growth may occur by aerobic respiration and under anoxic dark conditions, also by denitrification, fermentation, and oxidant-dependent metabolism**. Polysaccharides, poly-β-hydroxybutyric acid and polyphosphates may be present as storage products. Growth factors are required.

Mesophilic freshwater bacteria with optimal growth at neutral pH and in the absence of NaCl.

Habitats: freshwater environments providing organic substrates and reduced oxygen concentrations; sewage ponds, eutrophic lakes and the like.

The mol% G + C of the DNA is: 64.4–73.2.

Type species: Rhodobacter capsulatus (Molisch 1907) Imhoff, Trüper and Pfennig 1984, 342 (*Rhodonostoc capsulatum* Molisch 1907, 23; *Rhodopseudomonas capsulata* (Molisch 1907) van Niel 1944, 92.)

FURTHER DESCRIPTIVE INFORMATION

Cells of all *Rhodobacter* species have similar shape and size but different tendencies to form capsules, slime, or chains. Chain formation is also dependent on the growth conditions. Characteristic for *R. capsulatus* is the formation of zigzag chains, which has not been observed in the other species. However, not all strains of this species form zigzag chains, and this phenomenon is best observed in mineral media. Most of the strains that form zigzag chains in mineral media form straight chains in complex media, in which a marked tendency of spheroplast formation was observed, especially if the concentrations of yeast extract were higher than 0.7% (Weaver et al., 1975a).

Physiologically, *Rhodobacter* species are among the most versatile species of the purple nonsulfur bacteria. They can perform a number of different growth modes. Most of the biochemical and genetic research with purple nonsulfur bacteria has been performed with either of the two species, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. All species grow well photoheterotrophically under anoxic conditions in the light with a variety of

carbon sources and electron donors. Most species grow photoautotrophically with hydrogen (except *R. veldkampii*). Molecular hydrogen is an excellent electron donor for *R. capsulatus*, while *R. sphaeroides* grows only slowly with hydrogen (Klemme, 1968; Gest et al., 1983). Sulfide is used as a photosynthetic electron donor in all species but *R. blasticus*. The oxidation product of sulfide is elemental sulfur in most species; only in *R. veldkampii* is sulfate the final oxidation product, and elemental sulfur appears as an intermediate outside the cells (Hansen et al., 1975). Thiosulfate and elemental sulfur are used only by *R. veldkampii*. Sulfate is assimilated via 3'-phosphoadenosine-5'-phosphosulfate (PAPS) in those species which are able to use sulfate as sole sulfur source (Imhoff, 1982). *R. veldkampii* lacks this capability and depends on reduced sulfur compounds for growth.

R. capsulatus can grow well under chemoautotrophic conditions aerobically in the dark with molecular hydrogen as electron donor (Madigan and Gest, 1979; Siefert and Pfennig, 1979). Under oxic dark conditions, it can also use sulfide as electron donor (Kompantseva, 1981). Under anoxic dark conditions, pyruvate and sugars can be fermented (Gürgün et al., 1976; Schultz and Weaver, 1982). Sugars, as well as succinate, malate, or acetate, can support anaerobic dark growth of *R. capsulatus* if DMSO or trimethylamine-*N*-oxide is present as electron acceptor (Yen and Marrs, 1977; Madigan and Gest, 1978; Schultz and Weaver, 1982). Molar growth yields from cultures grown anaerobically in the dark with fructose and DMSO were about 60% of that obtained from aerobic respiratory growth with fructose (Schultz and Weaver, 1982) and about 4–5 times higher than those from cultures grown under anoxic dark conditions without DMSO.

Nitrate reductase-linked electron transport and membrane potential generation accompanied by nitrate reduction to nitrite have been found in strains of *R. capsulatus* (McEwan et al., 1983). Nitrite is accumulated and not reduced further. Some strains of *R. sphaeroides* have the ability to denitrify under anoxic dark conditions (Satoh et al., 1974, 1976; Pellerin and Gest, 1983). Denitrification is also found in *R. azotoformans* (Hiraishi et al., 1996).

The abilities to assimilate and dissimilate nitrate are not linked to each other. Denitrifying strains of *R. sphaeroides* are unable to use nitrate as an assimilatory nitrogen source (Satoh et al., 1976) but have the capacity to fix dinitrogen derived from denitrification (Kelley et al., 1982). Nitrate is assimilated under anoxic conditions in the light by strains of *R. capsulatus*, *R. sphaeroides* (Klemme, 1979), and *R. veldkampii* (Hansen et al., 1975).

Ammonia is certainly the preferred nitrogen source. It is assimilated via the glutamine synthetase/glutamate synthase (NADPH-dependent) reactions (Brown and Herbert, 1977) in *R. capsulatus* and *R. sphaeroides*. A nucleotide-unspecific glutamate dehydrogenase is present in *R. sphaeroides* (Engelhardt and Klemme, 1978), but absent in *R. capsulatus*, in which an alanine dehydrogenase, which acts primarily in the catabolism of alanine, was found (Johansson and Gest, 1976; Tolxdorff-Neutzling and Klemme, 1982).

Besides ammonia, dinitrogen is commonly used as a nitrogen source by *Rhodobacter* species (Siefert, 1976; Madigan et al., 1984). Nitrogen fixation is possible not only under anoxic conditions in the light but also under microoxic to oxic conditions in the dark (Madigan et al., 1979; Siefert and Pfennig, 1980) and under anoxic denitrifying conditions in strains of *R. sphaeroides* (Kelley et al., 1982). A number of organic nitrogen compounds also serve as sources for cellular nitrogen, including purines (Aretz et al., 1978) and pyrimidines (Kaspari, 1979).

Most species are sensitive to penicillin (Weaver et al., 1975a;

de Bont et al., 1981a; Hansen and Imhoff, 1985). With the exception of *R. sphaeroides*, growth inhibition is almost complete at 0.1 U/ml penicillin. Growth of *R. sphaeroides* is completely inhibited at 1–100 U/ml of penicillin.

ENRICHMENT AND ISOLATION PROCEDURES

Standard media and techniques for enrichment and isolation of PNSB are suitable for *Rhodobacter* species (see Genus *Rhodospirillum*). In enrichment cultures set up for purple nonsulfur bacteria, members of this genus will usually grow faster and outcompete other purple nonsulfur bacteria. Media for the enrichment of *Rhodobacter* species should not contain NaCl in order to specifically exclude *Rhodovulum* species. Media with organic substrates and for most species, the provision of autotrophic conditions with sulfide, thiosulfate, or hydrogen as electron donors are equally effective for enrichment of *Rhodobacter* species (Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Trüper, 1992). Photoautotrophic conditions with hydrogen and dinitrogen as electron and nitrogen sources, respectively, appear highly selective for *R. capsulatus*.

MAINTENANCE PROCEDURES

Cultures are well preserved by use of standard techniques with liquid nitrogen, by lyophilization, or storage at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOBACTER* FROM OTHER GENERA

Characteristic properties of *Rhodobacter* species are the ovoid to rod-shaped cell morphology, the presence of vesicular internal membranes (except in *R. blasticus*), and carotenoids of the spheroidene series. All these properties are shared with *Rhodovulum* species. In addition, *Rubrivivax gelatinosus* (*Betaproteobacteria*) forms carotenoids of the spheroidene series. *Rhodobacter* species can be distinguished from *Rhodovulum* species by the lack of a substantial NaCl requirement for optimal growth, i.e., they show the typical response of freshwater bacteria. This does not exclude, however, very minor requirements for the sodium ion that have been demonstrated e.g., for *R. sphaeroides*, which has a growth optimal at 4 mM sodium chloride (Sistrom, 1960).

Rhodobacter species have a number of characteristic chemotaxonomic properties that enable their distinction from other genera. All investigated species have a large-type cytochrome c_2 (Ambler et al., 1979) and as sole quinone component Q-10 (Imhoff, 1984a). Those species that are able to assimilate sulfate use the pathway via 3'-phosphoadenosine-5'-phosphosulfate (PAPS; Imhoff, 1982). The lipopolysaccharides of investigated species (*R. capsulatus*, *R. sphaeroides*, *R. blasticus*, *R. veldkampii*) contain glucosamine as sole amino sugar in their lipid A moieties, have phosphate, amide-linked 3-OH-14:0 and/or 3-oxo-14:0 and ester-linked 3-OH-10:0 (Weckesser et al., 1995). All of these properties are shared with *Rhodovulum* species. Differentiation of the genera and species of *Rhodobacter*, *Rhodobaca* and *Rhodovulum* is possible based on 16S rDNA sequences (see Fig. 3 [p. 129] in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A), by phenotypic characteristics (see Tables BXII.α.46, BXII.α.47, and BXII.α.48), and by DNA–DNA hybridization.

TAXONOMIC COMMENTS

Species of this genus (*R. capsulatus*, *R. sphaeroides*, *R. blasticus*) were formerly included in the genus *Rhodopseudomonas*. The rec-

TABLE BXII.α.46. Differentiating characteristics of the genera *Rhodobacter*, *Rhodobaca*, and *Rhodovulum*^a

Characteristic	<i>Rhodobacter</i>	<i>Rhodobaca</i>	<i>Rhodovulum</i>
Salt required for optimal growth	—	+	+
Optimal pH	6.5–7.5	9.0	6.5–7.5
Final oxidation product of sulfide	S ⁰ /SO ₄ ²⁻	S ⁰	SO ₄ ²⁻
<i>Utilization of:</i>			
Formate	+ / —	—	+
Thiosulfate	+ / —	nd	+
<i>Polar lipid composition:</i>			
Phosphatidylcholine	+ / —	nd	—
Sulfolipid	+ / —	nd	+
Mol% G + C of genomic DNA	64–70	58.8	62–69
Light-harvesting complexes	LHI and LHII	LHI	LHI and LHII
Natural habitat	Freshwater and terrestrial environments	Soda lakes	Hypersaline and marine environments
<i>16S rRNA signature(s) at position(s):^b</i>			
359	G	G	A
408	C	C	C
578	A	G	G
1311	G	G	C
1353–1355	CGT	CGG	CGT
1365–1367	ACG	CCG	ACG
1473	G	G	A
1449–1452	GCAA	CAAT	TTC/AG

^aSymbols: +, positive in most strains; —, negative in most strains; + / —, variable in different strains; nd, not determined.^bNucleotide position numbers are those of the *E. coli* numbering system.**TABLE BXII.α.47.** Differentiating characteristics of *Rhodobacter* species^a

Characteristic	<i>Rhodobacter capsulatus</i>	<i>Rhodobacter azotoformans</i>	<i>Rhodobacter blasticus</i>	<i>Rhodobacter sphaeroides</i>	<i>Rhodobacter veldkampii</i>
Cell diameter (μm)	0.5–1.2	0.6–1.0	0.6–0.8	2.0–2.5	0.6–0.8
Motility	+	+	—	+	—
<i>Internal membrane system:</i>					
Vesicle	+	+		+	+
Lamellae			+		
<i>Cell division:</i>					
Binary fission	+	+		+	+
Budding			+		
Slime production	±	+	—	±	—
NaCl required	— ^b	— ^b	—	— ^b	—
Sulfate assimilated	+	+	+	+	—
Oxidation products of sulfide	S ⁰	S ⁰	—	S ⁰	Sulfate
Aerobic dark growth	+	+	+	+	+
<i>Anaerobic growth with:</i>					
Nitrate (denitrification)	—	+	—	±	—
Dimethylsulfoxide	+	—	nd	+	nd
Trimethylamine-N-oxide	+	—	nd	+	nd
Vitamins required ^c	t (b, n)	b, n, t	b, n, t, B ₁₂	b, t, n	b, <i>p</i> -ABA, t
<i>Utilization of:</i>					
Formate	+	+	—	—	—
Citrate	—	nd	+	+	—
Tartrate	—	—	—	+	—
Mannitol	±	+	+	+	—
Glycerol	—	+	+	+	—
Ethanol	—	nd	—	+	—
Hydrogen	+	nd	+	+	—
Thiosulfate	—	—	—	—	+
Mol% G + C of DNA	65.5–66.8 (Bd), 68.1–69.6 (<i>T_m</i>)	69.5–70.2 (HPLC)	65.3 (Bd)	68.4–69.9 (Bd), 70.8–73.2 (<i>T_m</i>)	64.4–67.5 (<i>T_m</i>)

^aSymbols: +, positive; —, negative; ±, variable in different strains; nd, not determined.^bOptimal growth in the absence of NaCl but able to grow at 3% NaCl.^cb, biotin; B₁₂, vitamin B₁₂; n, niacin; *p*-ABA, *p*-aminobenzoic acid; t, thiamine; (b, n), a few strains require biotin and/or niacin.

ognition of morphologically and chemotaxonomically distinct characteristics and early phylogenetic analyses led to their separation from *Rhodopseudomonas* and classification in the genus *Rhodobacter* (Imhoff et al., 1984; Kawasaki et al., 1993a). *Rhodobacter blasticus* is the only species of this genus that forms buds during

cell division and contains internal photosynthetic membranes of the lamellar type (Eckersley and Dow, 1980). Because these were characteristic properties of the genus *Rhodopseudomonas* at that time, this bacterium was described as a *Rhodopseudomonas* species (Eckersley and Dow, 1980) and retained within this genus in a

TABLE BXII.α.48. Photosynthetic electron donors and carbon sources of *Rhodobacter* species^a

Donor/source	<i>R. capsulatus</i>	<i>R. azotoformans</i>	<i>R. blasticus</i>	<i>R. sphaeroides</i>	<i>R. veldkampii</i>
Formate	+	+	—	—	—
Acetate	+	+	+	+	+
Propionate	+	+	+	+	+
Butyrate	+	+	+	+	+
Valerate	+	nd	nd	+	+
Caproate	+	nd	nd	+	+
Caprylate	+	—	nd	+	+
Pelargonate	—	nd	nd	+	+
Pyruvate	+	+	+	+	+
Lactate	+	+	+	+	+
Malate	+	+	+	+	+
Succinate	+	+	+	+	+
Fumarate	+	+	+	+	+
Tartrate	—	—	—	+	—
Citrate	±	nd	+	+	—
Aspartate	±	nd	nd	nd	+
Arginine	nd	nd	nd	nd	nd
Glutamate	+	+	+	+	+
Benzoate	—	—	—	—	—
Gluconate	—	nd	nd	+	nd
Glucose	+	+	+	+	+
Fructose	+	+	+	+	—
Mannose	—	+	nd	+	nd
Mannitol	±	+	+	+	—
Sorbitol	±	+	+	+	—
Glycerol	—	+	+	+	—
Methanol	—	—	—	±	—
Ethanol	—	nd	—	+	—
Propanol	+	nd	nd	nd	—
Hydrogen	+	nd	+	+	—
Sulfide	+	+	—	+	+
Thiosulfate	—	—	—	—	+
Sulfur	—	—	—	—	+

^aSymbols: +, positive in most strains; —, negative in most strains; ±, variable in different strains; (+) weak growth or microaerobic growth only; nd, not determined.

reclassification of the purple nonsulfurbacteria because of insufficient supporting data (Imhoff et al., 1984). With the availability of 16S rDNA sequences and supporting chemotaxonomic data (e.g., lipopolysaccharide analyses; see Weckesser et al., 1995), the great similarity and phylogenetic affiliation of this species to *Rhodobacter* became apparent and led to its reclassification as *Rhodobacter blasticus* (Kawasaki et al., 1993a). These authors correctly proposed *Rhodobacter blasticus* comb. nov. as the new name but did not give a formal description of the new combination. Unfortunately, this name appeared incorrectly in

validation list no. 51 of the IJSB as “*Rhodobacter blastica*” and therefore a revision of this name was proposed by Euzéby (1997). As the genus name *Rhodobacter* is masculine, the correct name is *R. blasticus*.

Strains of *R. sphaeroides* that were confirmed as this species by DNA–DNA hybridization studies (de Bont et al., 1981a) are able to denitrify (Sato et al., 1976; Pellerin and Gest, 1983). The denitrifying *Rhodobacter azotoformans* is clearly distinct from *R. sphaeroides* by DNA–DNA hybridization (Hiraishi et al., 1996).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOBACTER*

The apparent morphological similarity, highly similar pigmentation, and great intraspecies variability regarding the utilization of carbon sources demands careful analyses to identify and differentiate *Rhodobacter* species. Problems in species identification that may arise due to high metabolic versatility and flexibility of the *Rhodobacter* species have been discussed in regard to the differentiation of *R. sphaeroides* and *R. capsulatus* (Imhoff, 1989). Characteristic properties to differentiate species of *Rhodobacter* are summarized in Tables BXII.α.47 and BXII.α.48. The utilization of formate, citrate, tartrate, mannitol, glycerol, ethanol,

hydrogen, and thiosulfate is of diagnostic value (Table BXII.α.48).

Rhodobacter species are well characterized and clearly distinct based on 16S rDNA sequence comparison (Fig. 3 (p. 129) of the introductory chapter “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A) and by DNA–DNA hybridization (de Bont et al., 1981a; Ivanova et al., 1988; Hiraishi et al., 1996). In addition, all species of *Rhodobacter* (and *Rhodovulum*) that were included in a study of fatty acid and polar lipid composition can be differentiated on this basis (Imhoff, 1991; Imhoff and Bias-Imhoff, 1995).

List of species of the genus *Rhodobacter*

1. ***Rhodobacter capsulatus*** (Molisch 1907) Imhoff, Trüper and Pfennig 1984, 342^{VP} (*Rhodonostoc capsulatum* Molisch 1907,

- 23; *Rhodopseudomonas capsulata* (Molisch 1907) van Niel 1944, 92.)

cap.su.la'tus. L. dim. n. *capsula* a small chest, capsule; M.L. masc. adj. *capsulatus* capsuled.

Cells are ovoid to rod-shaped, $0.5\text{--}1.2 \times 2.0\text{--}2.5 \mu\text{m}$, sometimes even longer. Spherical cells occur in media below pH 7.0 and are often irregularly arranged in chains resembling streptococci. Ovoid and rod-shaped cells are characteristic in media above pH 7.0, but pleomorphic cells appear and media become mucoid above pH 8.0. Chains of cells in zigzag arrangement are typical, but in some strains, these chains may be straight. Most strains that show zigzag arrangement in mineral media form straight chains in complex media. Capsules of varying thickness are formed. Cells are motile by means of polar flagella. Internal photosynthetic membranes appear as vesicles.

Cultures grown anaerobically in the light are yellowish brown or greenish to deep brown. When grown in the presence of oxygen, they are red. Anaerobically grown cells change their color to a distinct red when shaken with air for a few hours; light enhances the color change. Absorption spectra of living cells show maxima at 376–378, 450–455, 478–480, 508–513, 590–592, 802–805, and 860–863 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series, including spheroidene and hydroxyspheroidene. These two carotenoids are converted to the corresponding ketocarotenoids under oxic conditions, which causes the color change to red.

Photoautotrophic growth with molecular hydrogen as electron donor is excellent, but is also possible with sulfide, which is oxidized to elemental sulfur only; thiosulfate and elemental sulfur are not used. Photoheterotrophic growth occurs anaerobically in the light with a variety of organic compounds. Chemotrophic growth aerobically in the dark occurs heterotrophically with organic substrates or autotrophically with molecular hydrogen at the full oxygen tension of air. With sugars, anaerobic dark growth occurs in the presence of DMSO or trimethylamine-*N*-oxide as oxidant. Some strains may use nitrate as electron acceptor under similar conditions, reducing it to nitrite. Marginal growth is possible during fermentation of pyruvate or sugars. Carbon sources utilized are listed in Table BXII.α.48.

Ammonia, dinitrogen, and a number of amino acids are used as nitrogen sources; nitrate only by some strains; guanine, xanthine, uric acid, cytidine, uracil, thymine, and adenine only under oxic conditions. Sulfate assimilation occurs. Thiamine is required as a growth factor; some strains also require biotin or biotin and niacin.

All strains of *R. capsulatus* are susceptible to lysis by at least 1 of 16 bacteriophages, which are species specific and do not attack other purple nonsulfur bacteria (Wall et al., 1975).

Mesophilic freshwater bacteria with optimal growth at pH 7.0 (range: 6.5–7.5), 30–35°C (temperature maximum for most strains is above 36°C) and in the absence of added salt (NaCl).

Habitat: stagnant waters exposed to the light and with reduced oxygen concentrations, sewage plants, eutrophic ponds and lakes, and the like.

The mol% G + C of the DNA is: 65.5–66.8 (Bd) and 68.1–69.6 (T_m).

Type strain: ATCC 11166, ATH 2.3.1, DSM 1710, NCIB 8286.

GenBank accession number (16S rRNA): D16428, D134.

2. **Rhodobacter azotoformans** Hiraishi, Muramatsu and Ueda 1997a, 601^{VP} (Effective publication: Hiraishi, Muramatsu and Ueda 1996, 175.)

a.zo.to.for'mans. Fr. n. *azote* nitrogen; L. part. adj. *formans* forming; M.L. adj. *azotoformans* nitrogen forming.

Cells are ovoid to rod-shaped, $0.6\text{--}1.0 \times 0.9\text{--}1.5 \mu\text{m}$, motile by means of single polar flagella and divide by binary fission. Pleomorphic and swollen cells occur when growing in the presence of peptone or yeast extract at a concentration of 0.1% or more. Slime is produced under phototrophic growth conditions. Neither zigzag arrangement of cells in chains nor rosette formation is found. Internal photosynthetic membranes appear as vesicles. The color of phototrophic cultures is yellow-green to yellow-brown, while aerobic cultures are pink to red. Phototrophically grown cells have absorption maxima at 376, 449, 476, 510, 589, 800, and 850 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series.

Growth occurs under anoxic conditions in the light and under oxic conditions in the dark at full atmospheric oxygen tension. Sulfide at low concentrations (less than 0.5 mM), but not thiosulfate, is utilized under phototrophic conditions and oxidized to elemental sulfur as the final oxidation product. Anaerobic growth in darkness occurs with nitrate (by denitrification) but not with dimethylsulfide or trimethylamine *N*-oxide as a terminal electron acceptor. Photoheterotrophy with various organic compounds is the preferred mode of growth. Good carbon sources are acetate, pyruvate, lactate, succinate, fumarate, malate, D-xylulose, D-fructose, D-glucose, D-mannose, D-mannitol, L-alanine, L-asparagine, L-glutamate, peptone, Casamino acids, and yeast extract. Growth also occurs with formate, propionate, butyrate, malonate, D-dulcitol, D-sorbitol, glycerol, and L-leucine. Not utilized are caprylate, tartrate, glycolate, benzoate, and methanol. Nitrogen sources are ammonia, dinitrogen, and glutamate. Nitrogenase-dependent photoevolution of hydrogen gas is found under ammonium-limited conditions. Sulfate is assimilated. Biotin, niacin, and thiamine are required as growth factors.

Mesophilic freshwater bacterium with optimal growth at pH 7.0–7.5, 30–35°C and in the absence of sodium chloride. Growth is possible at 3% NaCl.

Habitat: sewage and activated sludge of wastewater treatment plants.

The mol% G + C of the DNA is: 69.5–70.2 (HPLC).

Type strain: KA25, JCM 9340.

GenBank accession number (16S rRNA): D70846.

3. **Rhodobacter blasticus** (Eckersley and Dow 1981) Kawasaki, Hoshino, Hirata and Yamasato 1994a, 852^{VP} (Effective publication: Kawasaki, Hoshino, Hirata and Yamasato 1993a, 362.) (*Rhodopseudomonas blastica* Eckersley and Dow 1981, 216.)

blas'ti.cus. Gr. adj. *blasticos* to bud; M.L. masc. adj. *blasticus* apt to bud.

Cells are ovoid to rod-shaped, $0.6\text{--}0.8 \times 1.0\text{--}2.5 \mu\text{m}$, nonmotile, reproduce by budding, and form sessile buds. Although mother and daughter cells appear morphologically similar at cell division, they differ with regard to the

time taken to reach the next division. The mother cell initiates cell growth immediately after division, whereas the daughter cell has to undergo an obligate period of maturation. In media containing yeast extract, abnormally swollen cells and spheroplasts are formed. Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane.

Color of cell suspensions is orange-brown if grown under anoxic conditions in the light, but changes to red in the presence of oxygen. Cells grown under oxic conditions in the dark are colorless to faint pink. Absorption spectra of living cells have maxima at 378, 418, 476, 506, 590, 795, and 862 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Preferred growth is photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth occurs with hydrogen as electron donor; sulfide and thiosulfate are not used. Chemotrophic growth is possible under oxic conditions in the dark. Ammonia, dinitrogen, glutamate, aspartate, glutamine, alanine, ornithine, tyrosine, thymine, and urea serve as nitrogen sources; nitrate and nitrite are not used. Sulfate is assimilated. In addition to thiamine and niacin, biotin and vitamin B₁₂ are required as growth factors (Schmidt and Bowien, 1983).

Mesophilic freshwater bacterium with optimal growth at pH 6.5–7.5, 30–35°C and in the absence of salt (NaCl).

Habitat: stagnant waters exposed to the light and with reduced oxygen concentrations, sewage plants, eutrophic ponds, and lakes.

The mol% G + C of the DNA is: 65.3 (Bd).

Type strain: NCIB 11576, ATCC 33485.

GenBank accession number (16S rRNA): D16429, D138.

4. ***Rhodobacter sphaeroides*** (van Niel 1944) Imhoff, Trüper and Pfennig 1984, 342^{VP} (*Rhodospseudomonas sphaeroides* van Niel 1944, 95.)

sphae.ro'i.des. Gr. adj. *sphaeroides* spherical.

Cells have highly variable morphology, especially in media containing complex nutrients; morphology is more uniform in mineral salts media (Fig. BXII.α.69). Cells are spherical to ovoid, 2.0–2.5 × 2.5–3.5 μm in sugar-containing media, 0.7–4.0 μm wide under other conditions. Frequently cells occur in pairs or as a chain of beads which in many instances are connected by a thin filament and slightly unequal in size. As cultures age, they become viscous due to slime production, except when sugars serve as carbon source. Slime formation is enhanced in complex media. In young cultures, cells are motile by polar flagella; motility ceases in alkaline media. Internal photosynthetic membranes appear as vesicles.

Cultures grown under anoxic conditions in the light are greenish brown to dark brown, if grown in the presence of oxygen they are red. The brown color of cells grown anaerobically in the light changes to red if cells are shaken with air; light stimulates this change. Absorption spectra of living cells have maxima at 372–375, 446–450, 474–481, 507–513, 588–590, 800–805, 850–852, and 870–880 nm. The relative absorbances at 850–880 nm vary greatly with the culture conditions. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series, including spheroidene and hydroxyspheroidene, which are converted to their corre-

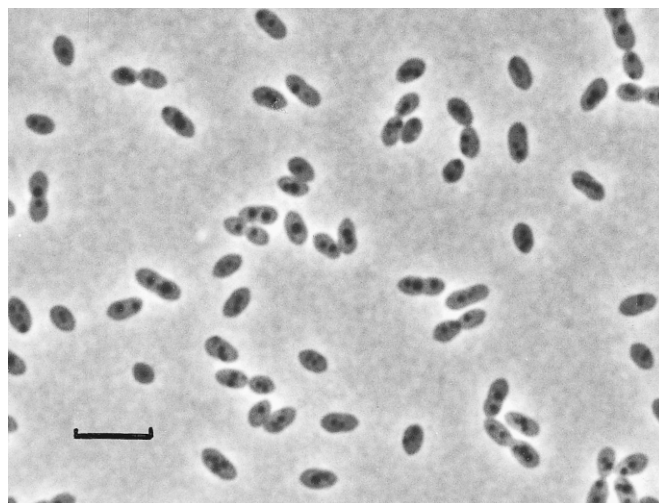


FIGURE BXII.α.69. *Rhodobacter sphaeroides* ATCC 17032 grown in mineral medium with 0.2% succinate and 0.05% yeast extract at pH 6.8. Phase-contrast micrograph. Bar = 5 μm. (Courtesy of N. Pfennig.)

sponding ketocarotenoids under oxic conditions and thereby cause the color change to red.

Preferred growth is photoheterotrophically under anoxic conditions. Photoautotrophic growth with molecular hydrogen or sulfide as electron donor is slow; thiosulfate and elemental sulfur are not used; sulfide is oxidized to elemental sulfur only. Good growth is observed under oxic conditions in the dark with a number of organic carbon sources. In addition, oxidant-dependent anaerobic dark growth with DMSO and TMAO as electron acceptors and several organic substrates is possible. Anaerobic dark fermentative growth with pyruvate and sugars is only marginal. Some strains are able to denitrify under anoxic conditions in the dark.

Organic substrates utilized are shown in Table BXII.α.48. Lower fatty acids are used at low concentrations. Higher fatty acids are toxic. Growth with glycerol depends on the presence of CO₂. Acids are produced in sugar-containing media under all conditions but disappear later. Nitrogen sources are ammonia, dinitrogen, alanine, glutamate, and aspartate; dinitrogen is also used under microoxic to oxic growth conditions in the dark. Some strains use uric acid, guanine, xanthine, cytidine, uracil, thymine, adenine (only under oxic conditions), and nitrate. Sulfate is assimilated. Thiamine, biotin, and niacin are required as growth factors.

Mesophilic freshwater bacterium with optimal growth at pH 7.0 (range: 6.0–8.5), 30–34°C, and in the absence of NaCl. Growth is possible at 3% NaCl.

Habitat: stagnant waters exposed to the light and with reduced oxygen concentrations, sewage plants, eutrophic ponds, and lakes.

The mol% G + C of the DNA is: 68.4–69.9 (Bd) and 70.8–73.2 (*T_m*).

Type strain: ATCC 17023, ATH 2.4.1, DSM 158, NCIB 8253.

GenBank accession number (16S rRNA): X53853, X53854, X53855.

5. ***Rhodobacter veldkampii*** Hansen and Imhoff 1985, 115^{VP}

veldkampii i.i. M.L. gen. n. *veldkampii* of Veldkamp; named for H. Veldkamp, a Dutch microbiologist.

Cells are ovoid to rod-shaped, $0.6\text{--}0.8 \times 1.0\text{--}1.3 \mu\text{m}$, nonmotile, have a pronounced tendency to form chains of cells under certain growth conditions, and do not produce slime. Internal photosynthetic membranes appear as vesicles. Color of photosynthetically grown cells varies from yellowish brown to dark brown and red. Aerobically grown cells are red. Absorption spectra of photosynthetically grown cells have maxima at 373, 448, 477, 510, 589, 803, and 855 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Photoheterotrophic growth occurs under anoxic conditions in the light with a variety of carbon compounds, if a reduced sulfur source is provided. Sulfide, thiosulfate, and elemental sulfur, but not hydrogen, serve as electron donors for photoautotrophic growth. Chemotrophic growth occurs aerobically in the dark. During phototrophic growth with sulfide, elemental sulfur is deposited outside the cells and

oxidized further to sulfate after sulfide depletion in batch cultures. In sulfide-limited chemostat cultures, sulfate is the major oxidation product. High concentrations of sulfide (5 mM) are tolerated. The carbon compounds utilized are shown in Table BXII.α.48.

Nitrogen sources are ammonia, dinitrogen, and nitrate. Sulfate cannot be assimilated and reduced sulfur sources are required: sulfide, cysteine, and cystine are used, but not sulfite, thiosulfate, and methionine. Biotin, thiamine, and *p*-aminobenzoic acid are required as growth factors; 0.01% yeast extract can replace the vitamin requirement.

Mesophilic freshwater bacterium with optimal growth (with succinate as substrate and in the presence of cysteine) at pH 7.5, 30–35°C, and in the absence of NaCl.

Habitat: stagnant waters exposed to the light and with reduced oxygen concentrations, sewage plants, eutrophic ponds, and lakes.

The mol% G + C of the DNA is: 64.4–67.5 (T_m).

Type strain: Hansen 51, ATCC 35703.

GenBank accession number (16S rRNA): D137, D16421.

Genus II. *Ahrensia* Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208

NOEL R. KRIEG

Ahrensia i.a. M.L. dim ending *-ia*; M.L. fem. n. *Ahrensia* named after R. Ahrens, a German microbiologist, who contributed to the taxonomy of marine species of *Agrobacterium*.

Rods, $0.6\text{--}1.0 \times 2.0\text{--}4.0 \mu\text{m}$. Motile. Do not form spores. Gram negative. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Na^+ is required for growth.** Optimal growth occurs between 20 and 30°C; growth occurs at 5°C but not at 37°C. **Catalase and oxidase positive.** Nitrate is not reduced to nitrite or to gas. Gelatin, starch, chitin, and alginate are not hydrolyzed. Indole is not produced. The major fatty acid is $\text{C}_{18:1}$. The 3-hydroxy fatty acid is $\text{C}_{12:0\ 3\text{OH}}$. 2-Hydroxy fatty acids are absent. The major quinone is ubiquinone 10. The genus belongs to the class *Alphaproteobacteria*. Isolated from seawater.

The mol% G + C of the DNA is: 48.

Type species: ***Ahrensia kieliensis*** (ex Ahrens 1968) Uchino, Hirata, Yokota and Sugiyama 1999, 1 (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium kieliense* Ahrens 1968, 156.)

FURTHER DESCRIPTIVE INFORMATION

Some discrepancies regarding the phenotypic characteristics of *Ahrensia* have become evident. Although Uchino et al. (1998) defined the genus *Ahrensia* as having polar flagella, they also indicated in a table that *A. kieliensis* possessed peritrichous flagella. Rüger and Höfle (1992) reported *A. kieliensis* to have peritrichous or degenerately peritrichous flagella.

It is also not clear whether *A. kieliensis* can use carbohydrates. Rüger and Höfle (1992) reported that none of the 34 compounds tested could be used as sole sources of carbon and energy after incubation at 20°C for 4 weeks. These compounds included glu-

cose and 11 other sugars, the salts of eight organic acids, glycerol, mannitol, sorbitol, and 7 amino acids. Acid production, however, was reported from glucose and xylose after 4–6 weeks of incubation at 20°C.

DIFFERENTIATION OF THE GENUS *AHRENSIA* FROM OTHER GENERA

It is not yet clear how the genus can be readily differentiated phenotypically from other aerobic marine rods such as *Alteromonas*, *Deleya*, *Marinomonas*, *Marinobacter*, *Neptunomonas*, *Oceanospirillum kriegii*, and *Oceanospirillum jannaschii*. It differs from the genus *Stappia* by having $\text{C}_{12:0\ 3\text{OH}}$ as its major 3-OH fatty acid rather than $\text{C}_{14:0\ 3\text{OH}}$.

TAXONOMIC COMMENTS

Ahrens (1968) classified a new marine species in the genus *Agrobacterium* as *Agrobacterium kieliense* but later withdrew this proposal (Allen and Holding, 1974). DNA–rRNA hybridization studies by De Smedt and De Ley (1977) showed that *Agrobacterium kieliense* was not an agrobacterium, although with a $T_{m(e)}$ of 72°C it did belong on the *Agrobacterium*–*Rhizobium*–*Phyllobacterium* branch. 16S rDNA sequence analysis by Uchino et al. (1998) showed that *Agrobacterium kieliense* was separated from other genera in the *Proteobacteria*, and the species was assigned to a new genus, *Ahrensia*, as *Ahrensia kieliense* (sic). In this edition of the *Manual*, the species is placed in the class *Alphaproteobacteria*, the order *Rhodobacterales*, and the family *Rhodobacteraceae*.

List of species of the genus *Ahrensia*

1. ***Ahrensia kieliensis*** (ex Ahrens 1968) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino,

Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium kieliense* Ahrens 1968, 156.)

kieliensis. M.L. fem. adj. *kieliensis* pertaining to Kiel, Germany.

The characteristics are as described for the genus. Isolated from brackish seawater of the Baltic Sea.

The mol% G + C of the DNA is: 48.

Type strain: B9, ATCC 25656, DSM 5980, IAM 12618, IFO 15762, NCMB 2205.

GenBank accession number (16S rRNA): D88524.

Genus III. *Amaricoccus* Maszenan, Seviour, Patel, Rees and McDougall 1997, 732^{VP}

ABDUL M. MASZENAN, ROBERT J. SEVIOUR AND BHARAT K.C. PATEL

A.ma'ri.coc.cus. Gr. n. *amara* sewage duct; Gr. n. *coccus* grain; L. n. *Amaricoccus* spherical cells from sewage ducts.

Cocci, 1.3–1.8 µm in diameter, usually arranged in tetrads. Gram negative. Nonmotile. Do not form spores or store polyphosphate granules. Oxidase positive. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Growth occurs at 20–37°C and at pH 5.5–9.0. Chemoheterotrophic. A variety of organic compounds can be used as carbon sources, including D-fructose, glucose, mannitol, L-glutamic acid, D-mannose, D-sorbitol, sucrose, L-rhamnose, L-arabinose, L-leucine, and L-histidine. Isolated from activated sludge and sewage.

The mol% G + C of the DNA is: 51–63.

Type species: *Amaricoccus kaplicensis* Maszenan, Seviour, Patel, Rees and McDougall 1997, 733.

FURTHER DESCRIPTIVE INFORMATION

The following enzymes are produced: alkaline phosphatase, esterase, esterase-lipase, lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase. Do not produce cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase.

The following compounds are used as carbon sources: dextrin, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, *m*-inositol, maltose, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, DL-lactic acid, succinic acid, succinamic acid, D-alanine, L-alanine, L-asparagine, L-glutamic acid, maltotriose, palatinose, D-ribose, salicin, D-tagatose, D-xylose, D-lactic acid methyl ester, L-lactic acid, D-malic acid, methyl succinate, pyruvic acid, N-acetyl-L-glutamic acid, and adenosine. The following compounds are not used: α-cyclodextrin, N-acetyl-D-galactosamine, *l*-erythritol, α-D-lactose, lactulose, D-raffinose, D-glucosaminic acid, D-saccharic acid, sebacic acid, L-alanyl-glycine, glycyl-L-aspartic acid, L-histidine, L-leucine, L-ornithine, L-pyroglutamic acid, uridine, inulin, amygdalin, α-methyl-D-galactoside, β-methyl-D-mannoside, sedoheptulosan, stachyose, and UMP.

Cech and Hartman (1993) suggested that “G-Bacteria” (*Amaricoccus* species) grew well in activated sludge systems operating with alternating aerobic/anaerobic zones, and were dominant when glucose was the carbon source rather than acetate. They hypothesized that “G-Bacteria” were detrimental to the process of biological phosphate removal as they outcompeted polyphosphate-accumulating bacteria (PAB) by anaerobic assimilation of glucose to the storage compound poly-β-hydroxybutyrate, which was subsequently used under aerobic conditions to form glycogen instead of polyphosphate. However, pure cultures of

Amaricoccus have failed to demonstrate any ability for anaerobic substrate utilization (Seviour et al., 2000).

Amaricoccus species have so far been isolated only from activated sludge. Data from whole-cell hybridization studies with a fluorescently labeled rRNA-targeted oligonucleotide probe suggest that *Amaricoccus* is widespread in the activated sludge environment (Maszenan et al., 2000).

ENRICHMENT AND ISOLATION PROCEDURES

Cech and Hartman (1990, 1993) initially isolated a pure culture of a bacterium described as “G-Bacteria” from a laboratory-scale activated sludge reactor sample. The isolate was subsequently shown to belong to a new genus *Amaricoccus* and described as *Amaricoccus kaplicensis* (Maszenan et al., 1997). A pure culture of “G-Bacteria” was also isolated using a Skerman micromanipulator and called “*Tetracoccus cechi*”, but because its 16S rRNA gene sequence was identical to that of *A. kaplicensis*, it was not taxonomically validated (Blackall et al., 1997). Maszenan et al. (1997, 1998) used a micromanipulator to isolate additional strains from activated sludge biomass samples onto a range of different media and found the Glucose Sulfide (GS) agar medium of Williams and Unz (1985)¹ to be the best for isolation purposes. Once isolated, pure culture cells were able to grow well on a range of media (Maszenan et al., 1997).

DIFFERENTIATION OF THE GENUS *AMARICOCCUS* FROM OTHER GENERA

Members of the order *Rhodobacterales* are heterogenous, comprising different morphologies and physiologies and varying in the mol% G + C value of the DNA. The genus *Amaricoccus* can be differentiated from phylogenetically closely related members of *Rhodobacterales* by the phenotypic criteria listed in Table BXII.α.49.

TAXONOMIC COMMENTS

Phylogenetic analyses based on 16S rDNA sequencing indicate that *Amaricoccus* species belong to the order *Rhodobacterales* in the class *Alphaproteobacteria* within the phylum *Proteobacteria* (Fig. BXII.α.70). The genus appears to be almost equidistant from *Paracoccus*, *Rhodobacter*, and *Rhodovulum* (91% similarity), and less closely related to *Roseobacter* (88% similarity).

The type strains of the four *Amaricoccus* species exhibit a high

1. The medium contains (g/l distilled water): glucose, 0.15; (NH₄)₂SO₄, 0.50; CaCO₃, 0.10; Ca(NO₃)₂, 0.10; KCl, 0.05; K₂HPO₄, 0.05; MgSO₄·7H₂O, 0.05; Na₂S·9H₂O, 0.187; 1.0 ml of vitamin solution (Eikelboom, 1975); agar technical No. 3 (Oxoid), 15; pH adjusted to 7.3 (± 0.2). The vitamin solution contains (µg/l): biotin, 5; calcium pantothenate, 100; cocarboxylase, 100; cyanocobalamin (vitamin B₁₂), 5; folic acid, 5; inositol, 100; niacin, 100; *p*-aminobenzoic acid, 100; pyridoxine, 100; riboflavin, 100; thiamine, 100.

TABLE BXII.α.49. Differential characteristics of the genus *Amaricoccus* and some closely related genera^{a,b}

Characteristic	<i>Amaricoccus</i>	<i>Paracoccus</i>	<i>Rhodobacter</i>	<i>Rhodovulum</i>	<i>Rubrimonas</i>
Cell size (μm)	1.3–1.8	1.0–1.3	0.5–1.2	0.6–1.0	1–1.5 × 1.2–2.0
<i>Cell shape:</i>					
Cocci	+ ^c				
Coccoid to short rods		+			
Ovoid to rod-shaped			+	+	
Small rods					+
Motile	–	–	+ ^d	+ ^d	+
<i>Mode of respiration:</i>					
Aerobic	+	+		+	+
Anaerobic			+	+	
Mol% G + C content of DNA	51–63	64–67	64–70	62–69	74.0–74.8
<i>Habitat:</i>					
Activated sludge	+				
Freshwater and terrestrial environments			+		
Marine or hypersaline environments				+	
Saline lake					+
Sewage	+	+			
Soil		+			
<i>Growth physiology:</i>					
Autotroph		+			
Chemoheterotroph	+	+			
Photoautotroph			+		
Photoheterotroph			+	+	+
<i>Carbon sources:</i>					
D-Fructose	+	+	V	+	+
Glucose	+	+	+	V	+
Mannitol	+	+	V	–	
L-Glutamic acid	+	+	+ ^e	–	–
D-Mannose	+	+		–	
D-Sorbitol	+	+		–	
Sucrose	+	+		–	–
L-Leucine	–	+		+	
L-Histidine	–	+			
L-Rhamnose	+	–			
L-Arabinose	+			w	+

^aSymbols: +, positive; –, negative; W, weakly positive; V, variable.^bModified from Maszenan et al. (1997) and Suzuki et al. (1999a).^cArranged in tetrads.^dHowever, nonmotile species have been reported.^e*Rhodobacter adriaticus* does not utilize L-glutamic acid.

level of 16S rDNA similarity (96.6%) to one another, but are deemed sufficiently different in their phenotypic characteristics to justify placement in separate species. A numerical taxonomic analysis of the phenotypic characteristics indicates that none of the type strains are linked to the others by an S_{sm} value greater than 0.85.

FURTHER READING

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *AMARICOCCUS*

Table BXII.α.50 lists the features that differentiate the four species of *Amaricoccus*.

List of species of the genus *Amaricoccus*

1. ***Amaricoccus kaplicensis*** Maszenan, Seviour, Patel, Rees and McDougall 1997, 733^{VP}

ka.pli.cen' sis. M.L. adj. *kaplicensis* referring to Kaplice, Czech Republic, the source of the type strain.

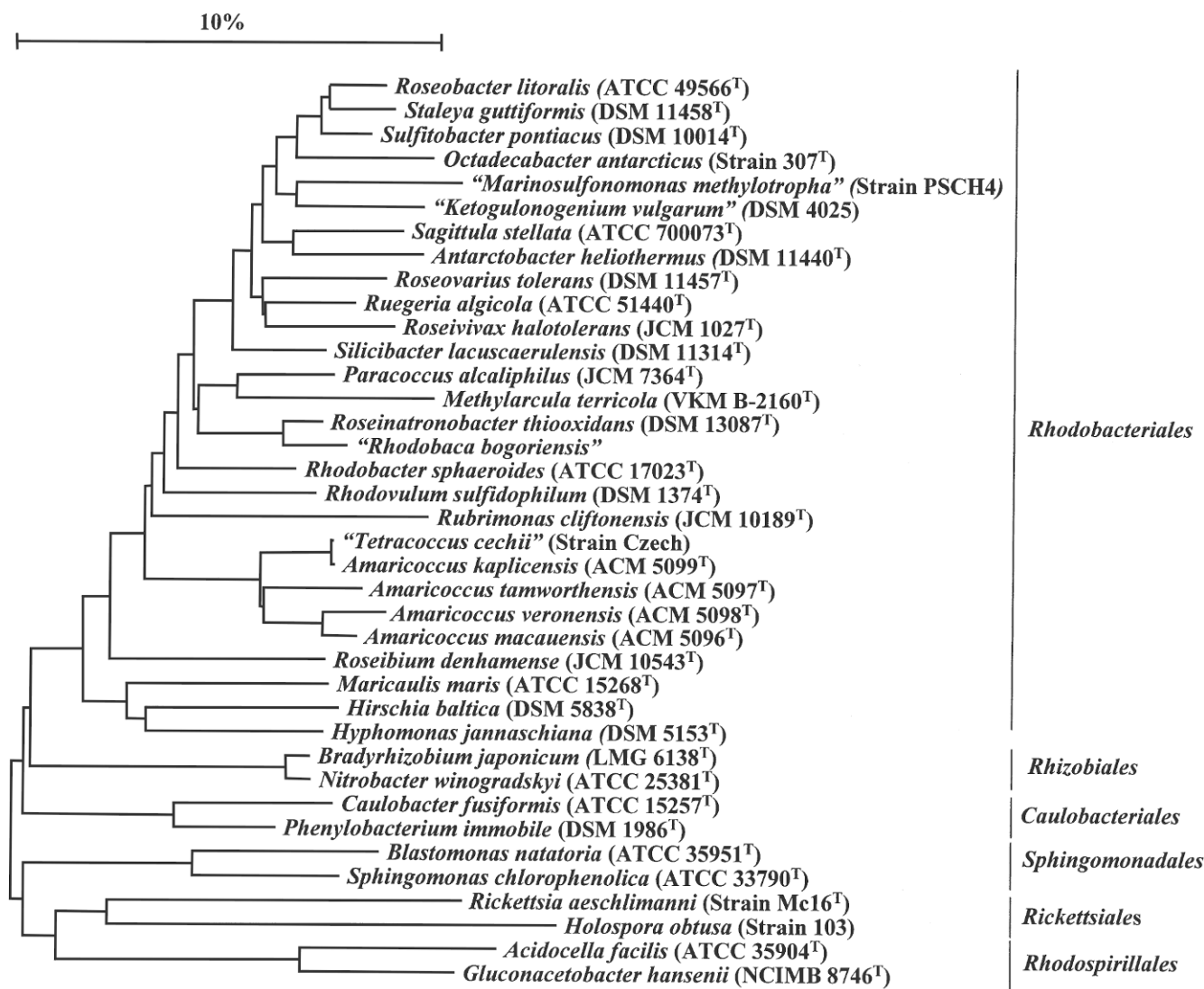


FIGURE BXII.α.70. The phylogenetic position of genus *Amaricoccus* as a member of the order *Rhodobacterales* is shown. The order *Rhodobacterales*, together with the orders *Rhizobiales*, *Caulobacteriales*, *Sphingomonadales*, *Rickettsiales*, and *Rhodospirillales*, constitute the class *Alphaproteobacteria*. The strains used in the analysis, their culture collection numbers and corresponding 16S rRNA gene sequences extracted from GenBank/EMBL are listed below. *Roseobacter litoralis* (X78312, ATCC 49566^T), *Staleyia guttiformis* (SY16427, DSM 11458^T), *Sulfitobacter pontiacus* (Y13155, DSM 10014^T), *Octadecabacter antarcticus* (U14583, strain 307^T), "*Marinosulfonomonas methylotrophus*" (U62894, strain PSCH4), "*Ketogulonogenium vulgare*" (AF136849, DSM 4025), *Sagittula stellata* (U58356, ATCC 700073^T), *Antarcticobacter heliothermus* (Y11552, DSM 11445^T), *Roseovarius tolerans* (Y11551, DSM 11457^T), *Ruegeria algicola* (X78315, ATCC 51440^T), *Roseivivax halotolerans* (D85831, JCM 1027^T), *Silicibacter lacuscaerulensis* (U77644, DSM 11314^T), *Paracoccus alcaliphilus* (Y17512, JCM 7364^T), *Methyloarcula terricola* (AF030437, VKM B-2160^T), *Roseinatronobacter thiooxidans* (AF249749, DSM 13087^T), *Rhodobaca bogoriensis* (AF248638^T), *Rhodobacter sphaeroides* (X53854, ATCC 17023^T), *Rhodovulum sulfidophilum* (U55277, DSM 1374^T), *Rubrimonas cliftonensis* (D85834, JCM 10189^T), "*Tetracoccus cecchi*" (Y09610^T, strain Czech), *Amaricoccus kaplicensis* (U88041, ACM 5099^T), *Amaricoccus tamworthensis* (U88044, ACM 5097^T), *Amaricoccus veronensis* (U88043, ACM 5098^T), *Amaricoccus macauensis* (U88042, ACM 5096^T), *Roseibium denhamense* (D85832, JCM 10543^T), *Maricaulis maris* (AJ227802, ATCC 15268^T), *Hirschia baltica* (X52909, DSM 5838^T), *Hyphomonas jannaschiana* (AF082789, DSM 5153^T), *Bradyrhizobium japonicum* (S46916, LMG 6138^T), *Nitrobacter winogradskyi* (L35506, ATCC 25381^T), *Caulobacter fusiformis* (AJ007803, ATCC 15257^T), *Phenyllobacterium immobile* (Y18216, DSM 1986^T), *Blastomonas natatoria* (X73043, ATCC 35951^T), *Sphingomonas chlorophenolica* (X87161, ATCC 33790^T), *Rickettsia aeschlimannii* (U74757, Strain Mc16^T), *Holospora obtusa* (endosymbiont), *Acidocella facilis* (D30774, ATCC 35904^T) and *Gluconacetobacter hansenii* (X75620, NCIMB 8746^T). Phylogenetic analysis was performed on 1230 unambiguous nucleotides using dnadist and neighbor-joining programs which form part of the PHYLIP suite of software. Bar = 10 nucleotide changes per 100 nucleotides. The following abbreviations have been used: ^T, Type culture; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; ACM, Australian Collection of Microorganisms; JCM, Japan Collection of Microorganisms; NCIMB, National Collection of Industrial and Marine Bacteria; VKM, All-Russian Collection of Microorganisms.

Pleomorphic cells, 1.6 µm in diameter. Colonies are mucoid on GS agar. D-galactosidic acid, lactone, and α-keto-valeric acid can be used as carbon sources. Does not utilize gentiobiose, β-methyl-D-glucoside, inositol, or lactamide. Nitrate reduced to nitrite.

The mol% G + C of the DNA is: 56 (*T_m*).

Type strain: Ben 101, ACM 5099.

GenBank accession number (16S rRNA): U88041.

TABLE BXII.α.50. Characteristics differentiating the species of the genus *Amaricoccus*^a

Characteristic	<i>A. kaplicensis</i>	<i>A. macauensis</i>	<i>A. tamworthensis</i>	<i>A. veronensis</i>
Average cell size (μm)	1.6	1.3	1.8	1.8
Mol% G + C content of DNA	56	63	51	55
<i>Growth on:</i>				
Glycogen	+	+	—	+
Tween 40	—	—	+	—
Tween 80	—	—	+	+
N-Acetyl-D-glucosamine	+	+	+	—
Adonitol	—	—	—	+
Gentiobiose	—	+	+	+
D-Melibiose	—	—	+	—
β-Methyl-D-glucoside	—	+	+	+
D-Glucuronic acid	—	+	—	—
Acetic acid	+	+	—	+
cis-Aconitic acid	+	+	—	—
Citric acid	—	+	—	—
L-Phenylalanine	—	+	—	—
L-Proline	—	+	—	+
α-Ketoglutaric acid	—	—	+	—
D-Serine	+	+	—	—
α-Ketobutyric acid	+	+	—	+
L-Threonine	—	+	—	+
D,L-Carnitine	—	+	—	+
γ-Aminobutyric acid	—	+	—	—
Urocanic acid	—	+	+	—
Inosine	+	+	—	+
Thymidine	+	+	—	+
Phenyl ethylamine	+	+	—	+
Formic acid	+	+	—	+
D-Galactonic acid	+	—	—	—
D-Galacturonic acid	—	—	+	—
D-Gluconic acid	+	+	+	—
γ-Hydroxybutyric acid	+	+	+	—
p-Hydroxyphenyl acetic	—	+	—	—
Itaconic acid	—	+	—	—
α-Ketovaleric acid	—	—	—	—
Malonic acid	—	+	—	—
Propionic acid	+	+	—	—
Quinic acid	—	+	—	—
Bromosuccinic acid	+	+	—	+
Glucuronamide	—	+	—	—
Alaninamide	+	+	—	+
L-Aspartic acid	+	+	—	+
Glycyl-L-glutamic acid	—	—	—	+
Putrescine	—	+	—	—
2-Aminoethanol	—	+	—	—
2,3-Butanediol	—	—	—	+
Glycerol	+	+	+	—
D,L-α-Glycerol phosphate	—	+	—	—
Glucose-1-phosphate	—	+	—	+
Glucose-6-phosphate	—	+	—	+
α-Cyclodextrin	—	—	+	+
Mannan	—	—	+	+
N-Acetylmannosamine	—	—	—	+
Arbutin	+	+	—	+
D-Melezitose	—	+	—	+
3-Methyl glucose	—	—	+	+
α-Methyl-D-glucoside	—	+	—	+
Lactamide	—	+	+	+
2-Deoxyadenosine	+	+	—	+
β-Methyl-D-galactoside	—	+	—	—
Fructose-6-phosphate	—	+	—	+
Hydroxy-L-proline	—	+	—	—
AMP	—	+	—	+
TMP	—	+	—	+

^aSymbols: see standard definitions.

2. ***Amaricoccus macauensis*** Maszenan, Seviour, Patel, Rees and McDougall 1997, 733^{VP}
ma.cau.en'sis. M.L. adj. *macauensis* referring to Macau, the source of the type strain.

Cells are 1.3 μm in diameter. D-Glucuronic acid, citrate, p-hydroxyphenylacetate, itaconate, malonate, quinic acid, glucuronamide, β-methyl-D-galactoside, hydroxy-L-proline, L-phenylalanine, γ-aminobutyric acid, putrescine, 2-ami-

noethanol, and DL- α -glycerol phosphate are used as carbon sources. Lysine decarboxylase activity is present.

The mol% G + C of the DNA is: 63 (T_m).

Type strain: BEN 104, ACM 5096.

GenBank accession number (16S rRNA): U88042.

3. **Amaricoccus tamworthensis** Maszenan, Seviour, Patel, Rees and McDougall 1997, 733^{VP}

tam.worth.en'sis. M.L. adj. *tamworthensis* referring to Tamworth, Australia, the source of the type strain.

Cells are 1.3 μ m in diameter. Individual tetrad cells are joined by an interconnecting fibril network. Tween 40, D-melibiose, D-galacturonic acid, and α -ketoglutaric acid can be used as carbon sources. Glycogen, acetic acid, formic acid, bromosuccinic acid, alaninamide, L-aspartic acid, inosine, thymidine, phenylethylamine, arbutin, and 2-deoxyadenosine are not used.

The mol% G + C of the DNA is: 51 (T_m).

Type strain: BEN 103, ACM 5097.

GenBank accession number (16S rRNA): U88044.

4. **Amaricoccus veronensis** Maszenan, Seviour, Patel, Rees and McDougall 1997, 733^{VP}

ve.ron.en'sis. M.L. adj. *veronensis* referring to Verona, Italy, the source of the type strain.

Cells are 1.8 μ m in diameter. Adonitol, glycyl-L-glutamic acid, 2,3-butanediol, N-acetylmannosamine, and α -methyl-D-glucoside can be used as carbon sources. N-Acetyl-D-galactosamine, D-gluconic acid, γ -hydroxybutyric acid, and glycerol are not used.

The mol% G + C of the DNA is: 58 (T_m).

Type strain: Ben 102, ACM 5098.

GenBank accession number (16S rRNA): U88043.

Genus IV. *Antarctobacter* Labrenz, Collins, Lawson, Tindall, Braker and Hirsch 1998, 1369^{VP}

MATTHIAS LABRENZ AND PETER HIRSCH

Ant.arc'to.bac.ter. M.L. *Antarctica* name of the South Pole continent; derived from Gr. pref. *anti* against (on the other side); Gr. adj. *arcticos* northern; M.L. masc. n. *bacter* Gr. n. *baktron* a rod or staff; *Antarctobacter* a rod-shaped bacterium from Antarctica.

Rods, one or both cell poles narrower, sizes vary due to age and growth conditions: 0.8–1.2 \times 2.0–33.6 μ m. Rosettes are formed. Gram negative. **Daughter cells may be motile.** No resting stages are known. **Aerobic**, with a strictly respiratory type of metabolism. **Intracellular granules of poly- β -hydroxybutyrate are present.** Cells have an absolute requirement for Na⁺. Temperature range <3–43.5°C, salinity range <1.0 to <10.0% NaCl and <10 to >150‰ of artificial seawater (ASW), pH range 5.3 to >9. **Oxidase and catalase positive.** Do not grow photoautotrophically with H₂/CO₂ (80:20) or photoorganotrophically with acetate or glutamate. Do not contain bacteriochlorophyll *a* (bchl *a*). Occur in marine habitats.

The mol% G + C of the DNA is: 62–63.

Type species: ***Antarctobacter heliothermus*** Labrenz, Collins, Lawson, Tindall, Braker and Hirsch 1998, 1371.

FURTHER DESCRIPTIVE INFORMATION

Visible aerobic growth of *A. heliothermus* appears after 3–5 d on medium PYGV (Staley, 1968) + 25‰ (or 40‰) artificial seawater (ASW; Lyman and Fleming, 1940) at 20°C. Colonies are circular, smooth, convex, 1–4 mm in diameter and brownish yellow. On R2A agar (Difco, Detroit, USA) + ASW the colonies are brownish red. Cell growth of *A. heliothermus* appears to be monopolar since one cell end is usually narrower and shorter, possibly indicating a budding process. In such cases, the “daughter cell” is often positioned at an angle to the “mother cell”. The cell sizes of the strains EL-54, EL-165, EL-185, and EL-219^T are very similar; a peculiar characteristic of laboratory-cultured cells, as well as those from the source (Ekho Lake, Vestfold Hills, East Antarctica), is the frequent formation of elongated cells up to 33 μ m in length. Motility is observed in some strains. Electron microscopy reveals 1–3 subpolar flagella in strains EL-54 and EL-185.

A. heliothermus has an absolute requirement for Na⁺, but none

for K⁺, Mg²⁺, Ca²⁺, Cl[−] or SO₄^{2−}. This species is susceptible to chloramphenicol, penicillin G, streptomycin, tetracycline, and polymyxin B. Nitrate is reduced to nitrite. Nitrite is not reduced aerobically, anaerobic reduction to N₂ is variable. Poly- β -hydroxybutyrate is formed intracellularly.

In the presence of available nitrogen, *A. heliothermus* uses succinate, butyrate, glutamate, acetate, pyruvate, malate, citrate, and α -D-glucose, but not methanol or methanesulfonic acid. In the absence of other N-sources, glutamate is utilized as a C- and N-source. Without any added nitrogen source, there occurs a slight growth with acetate, butyrate, pyruvate, malate, or succinate. Metabolism of carbon sources tested with the Biolog system was described by Labrenz et al. (1998).

Two strains of *A. heliothermus* (EL-54 and EL-165) grow microaerophilically, but they do not grow anaerobically in a photolithoautotrophic mode with H₂/CO₂ (80:20) in the gas phase or photoorganotrophically. Bacteriochlorophyll *a* is never found in cell suspensions nor in methanolic extracts. Absorption spectra of methanolic extracts are very similar to those of *Ruegeria algicola* (Lafay et al., 1995; Uchino et al., 1998) when grown on PYGV + 25‰ ASW.

The peptidoglycan of *A. heliothermus* contains *m*-diaminopimelic acid, and the peptidoglycan of strain EL-185 is of the directly cross-linked type A1 γ (Schleifer and Kandler, 1972). Respiratory quinone is Q-10. Phosphatidylglycerol and phosphatidylcholine are present as well as unknown phospholipids and an aminolipid. The dominant fatty acid is C_{18:1} (81.4–84.0%); other fatty acids are C_{12:1 3OH} (3.0–3.6%), C_{16:0} (0.6–1.1%), C_{16:1} (2.3–2.7%), C_{18:0} (0.7–1.4%), and C_{19:0 cyclo} (1.9–2.7%).

Comparative 16S rDNA sequence analysis shows that *A. heliothermus* is a member of the *Alphaproteobacteria*, specifically associated with the *Roseobacter* cluster of organisms, which includes *Sagittula*, *Octadecabacter*, *Silicibacter*, *Prionitis lanceolata* gall symbiont, *Roseobacter*, *Roseovarius*, *Ruegeria*, *Staleyia*, and *Sulfitobacter*.

ENRICHMENT AND ISOLATION PROCEDURES

For *A. heliothermus*, three enrichment procedures may be used: (1) 5 ml of the original water sample are inoculated into 10 ml of sterile medium "398" (Labrenz et al., 1998) and incubated for four weeks at 15°C under dim light (4.1 mmol photons m⁻² s⁻¹). (2) Original water samples (50 ml) are amended with 1 ml of a filter-sterilized solution of 12.5 mg/ml Bacto Yeast Extract in original sample water and incubated at 15°C and at 4.1 mmol photons m⁻² s⁻¹. (3) Original water samples (0.5 ml) are spread directly on agar plates of medium PYGV that have been prepared with original water of 10‰ salinity. Incubation at 15°C and in the dark yields the best results. Pure cultures are isolated by several dilution transfers on the corresponding agar media.

MAINTENANCE PROCEDURES

Strains of *A. heliothermus* are initially cultured on PYGV or R2A agar with 25 or 40‰ ASW. After incubation at 16–26°C to allow abundant growth, the cultures may be maintained in a refrigerator (4°C) for 6 months. They can be preserved indefinitely by lyophilization or in 15% glycerol (frozen at –72°C).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

For the study of 16S rRNA gene fragments, PCR amplification as described by Hudson et al. (1993) and Labrenz et al. (1998) is recommended.

DIFFERENTIATION OF THE GENUS *ANTARCTOBACTER* FROM OTHER GENERA

Table BXII.α.51 indicates those characteristics of *Antarctobacter* that differentiate it from other morphologically, physiologically, or chemotaxonomically similar organisms. Fig. BXII.α.71 presents an unrooted tree showing phylogenetic relationships of *Antarctobacter heliothermus* and closely related *Alphaproteobacteria*.

TAXONOMIC COMMENTS

The sum of respiratory lipoquinones, fatty acids, and polar lipid data indicates that *A. heliothermus* belongs to a group of organisms (at the genus or family rank) within the *Proteobacteria*.

Comparative 16S rDNA sequence analysis also shows that it is a member of the *Alphaproteobacteria* and specifically associated with the *Roseobacter* supercluster of organisms, which includes *Sagittula*, *Octadecabacter*, *Silicibacter*, *Prionitis lanceolata* gall symbiont, *Roseovarius*, *Ruegeria*, *Staleyia*, and *Sulfitobacter*. Sequence divergence values of >5% show this genus is genetically distinct from all currently recognized members of the *Proteobacteria*. Furthermore, bootstrap resampling shows that *A. heliothermus* does not possess a particularly significant phylogenetic affinity with any individual species within the above-mentioned genera.

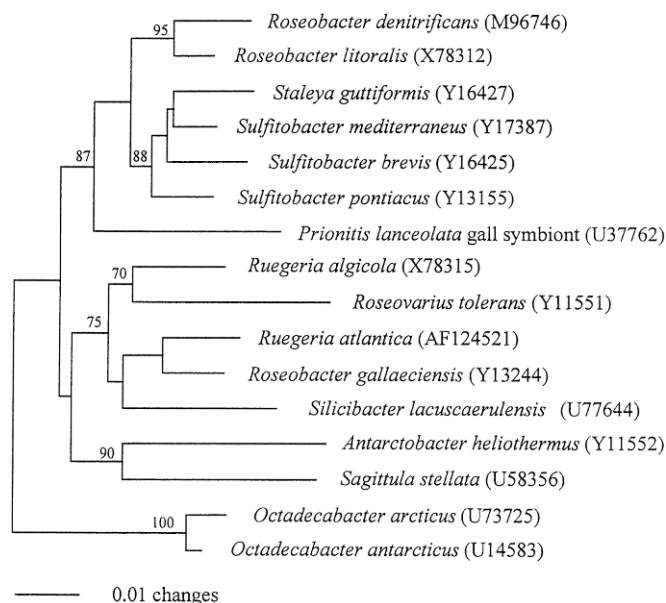


FIGURE BXII.α.71. Unrooted tree showing phylogenetic relationships of *Antarctobacter heliothermus* and closely related *Proteobacteria*. The tree was constructed using the neighbor-joining method; it was based on a comparison of approximately 1300 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points; only those above 70% are shown.

TABLE BXII.α.51. Differential characteristics of the genus *Antarctobacter* and other morphologically, physiologically, or chemotaxonomically similar organisms^a

Characteristic	<i>Antarctobacter</i>	<i>Octadecabacter arcticus</i>	<i>Roseobacter litoralis</i>	<i>Roseovarius tolerans</i>	<i>Sagittula stellata</i>	<i>Staleyia guttiformis</i>	<i>Sulfitobacter pontiacus</i>
Rosettes formed	+	–	–	–	+	+	+
Gas vesicles	–	+	–	–	–	–	–
Motile	d	–	+	d	+	+	+
Maximum length 10.9–33.6 μm	+	–	–	–	–	–	–
Growth at <8.5°C	+	+	+	+	+	+	+
Utilize methanol as carbon source	–	–	–	–	+	–	–
Oxidase activity	+	–	+	+	+	+	+
Cellulase activity	–	nd	nd	–	+	nd	nd
Bacteriochlorophyll <i>a</i>	–	–	+	d	–	V	–
Phosphatidylcholine	+	nd	–	+	nd	+	nd
Diphosphatidylglycerol	–	nd	+	+	nd	–	nd
Phosphatidylethanolamine	–	nd	–	+	nd	+	nd
C _{18:2} fatty acid	–	–	+	+	–	–	nd
C _{12:1} 3OH fatty acid	+	–	–	–	+	–	nd
Colony color brown-yellow	+	–	–	–	– ^b	–	–
Mol% G + C of DNA	62–63	57	56–58	63	65	55–56	62

^aFor symbols see standard definitions; nd, not determined; V, variable.

^bCream (González et al., 1997b).

List of species of the genus *Antarctobacter*

1. ***Antarctobacter heliothermus*** Labrenz, Collins, Lawson, Tindall, Braker and Hirsch 1998, 1371^{VP}
he.li.o.ther'mus. Gr. n. *helios* sun; Gr. adj. *thermos* hot; M.L. masc. n. *heliothermus* heated by the sun, referring to the heliothermal water layers of Ekho Lake, the source.

Rods with an average length of 3.8–7.7 µm. Further morphological description as for the genus. Physiological and nutritional characteristics of the species are presented in Table BXII.α.52. Optimal growth occurs at 16–26°C with salt concentrations of 20–60‰ NaCl or 10–70‰ ASW. Optimal pH for growth is 6.9–7.8. Colonies on medium PYGV + 25‰ (or 40‰) ASW are circular, smooth, convex, 1–4 mm in diameter and brownish yellow. Isolated from hypersaline, meromictic, and heliothermal Ekho Lake, East Antarctica.

The mol% G + C of the DNA is: 62–63 (HPLC).

Type strain: EL-219, DSM 11445.

GenBank accession number (16S rRNA): Y11552.

Additional Remarks: Reference strain: EL-165, DSM 11440.

TABLE BXII.α.52. Other characteristics of *Antarctobacter heliothermus*

Characteristic	<i>A. heliothermus</i>
Oxidase and catalase activity	+
NO ₃ [−] reduced to NO ₂ [−]	+
NO ₂ [−] reduced to N ₂	v
Requirement of thiamine or nicotinic acid	+
Requirement of pantothenate or vitamin B ₁₂	−
Stimulation by biotin	+
Hydrolysis of gelatin or DNA	+
Hydrolysis of Tween 80 or alginate	−
Hydrolysis of starch	d
Indole test	−
H ₂ S production	−
Voges–Proskauer test	−
Acid from glucose	−
Growth with succinate, glutamate, butyrate, acetate, citrate, pyruvate, malate, α-D-glucose	+
Growth with methanesulfonic acid	−
Susceptible to chloramphenicol, penicillin G, streptomycin, polymyxin B or tetracycline	+

Genus V. *Gemmobacter* Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1988, 328^{VP}
 (Effective publication: Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1987, 97)

PETER HIRSCH AND HEINZ SCHLESNER

Gem.mo.bac'ter. L. n. *gemma* a bud; M.L. masc. n. *bacter* equivalent; Gr. neut. n. *bactrum* a rod; M.L. masc. n. *Gemmobacter* a budding rod.

Rod-shaped cells with rounded poles or ovoid, 1.0–1.2 × 1.25–2.7 µm. New cell formation by budding from one cell pole or laterally (Fig. BXII.α.72). Daughter cells may stay connected with the mother cell resulting in formation of short chains. Not acid-fast, do not form spores or cysts; without prosthecae or other appendages. Nonmotile. **Gram negative.** Colonies colorless, round with entire margins. Temperature range 17–39°C, optimal temperature for growth 31°C.

Aerobic or facultatively anaerobic, chemoorganotrophic. **Sugars and ethanol are fermented.** Do not produce gas, D- or L-lactate, acetate or ethanol. **Dissimilatory nitrate reduction positive.** The respiratory ubiquinone is **Q-10**.

The mol% G + C of the DNA is: 63.4.

Type species: ***Gemmobacter aquatilis*** Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1988, 328 (Effective publication: Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1987, 98.)

FURTHER DESCRIPTIVE INFORMATION

Cells of *Gemmobacter* are morphologically and physiologically similar to those of members of the *Blastobacter* genus and *Sphingomonas natatoria* (Table BXII.α.53). Analysis by the 16S rRNA cataloging approach indicated that *Gemmobacter* is a member of the *Alphaproteobacteria* (Rothe et al., 1987).

ENRICHMENT AND ISOLATION PROCEDURES

The strain was isolated from water of a forest pond near Augusta (Michigan, USA). A 100-ml water sample was placed in a 250-ml Erlenmeyer flask and Bacto-peptone (Difco) was added to a final concentration of 0.005%. After plugging the flask with cotton,

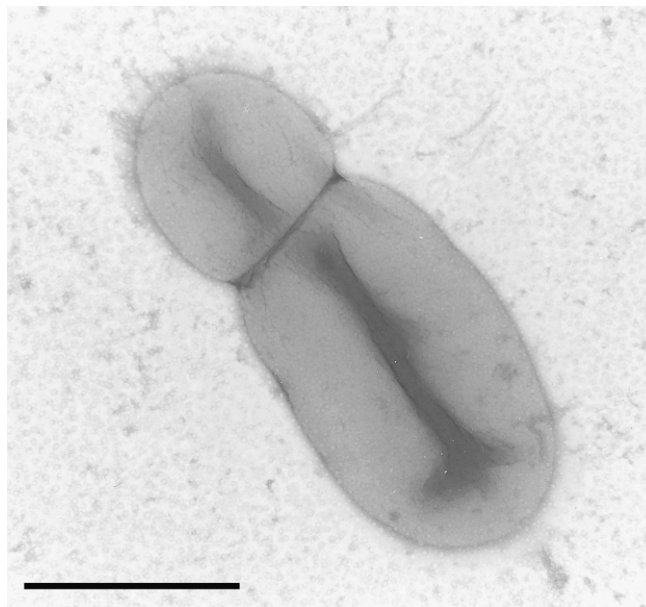


FIGURE BXII.α.72. Budding cell of *Gemmobacter aquatilis* IFAM 1031^T. Transmission electron micrograph of negatively stained cell. Bar = 1 µm.

TABLE BXII.α.53. Properties of diagnostic value for *Gemmobacter aquatilis*, *Blastobacter* spp., and *Sphingomonas natatoria*^a

Characteristic	<i>Gemmobacter aquatilis</i>	<i>Blastobacter aggregatus</i>	<i>Blastobacter capsulatus</i>	<i>Blastobacter denitrificans</i>	<i>Sphingomonas natatoria</i>
Cell shape	Ovoid to rods, short chains	Ovoid to rods	Rods, often bent	Rods with rounded poles	Rods with rounded end, slightly curved or wedge-shaped
Initial bud shape	Spherical or ovoid	Rods	Ovoid	Rods	Spherical or ovoid
Bud origin	Polar to subpolar	Distant pole	Distant pole or lateral	Slightly subpolar	Polar
Capsule formation	—	—	+	—	—
Motility	—	+	—	+	+
Rosette formation	—	+	—	—	+
Colony pigmentation	Colorless	Colorless	Colorless	Yellow	Yellow or pale pink
Utilization of sucrose	+	+	+	—	nd
N-Acetylglucosamine	—	—	—	+	nd
Succinate, malate, tartrate	+	—	—	+	nd
L-Arginine	+	+	—	—	nd
L-Proline	+	+	+	—	nd
Methanol (0.5% v/v)	—	—	—	+	—
Ethanol (0.4% v/v)	+	+	—	+	nd
Aerobically produce acid from glucose	+	+	+	—	+
Mol% G + C	63.4	60.4	58.9	64.5	65
Nicked 23S rRNA	+	—	+	—	nd
Hydroxy fatty acids, (rel. %): ^b					
C _{10:0} 3OH	23.7	—	—	—	—
C _{14:1} 2OH	—	—	—	—	0.6
C _{14:0} iso	—	—	—	—	52.2
C _{14:0} 3OH	—	1.2	0.4	nd	—
C _{14:0} 3OH	—	81.7	85	nd	—
C _{15:0} 2OH	—	—	—	—	3.9
C _{16:1} ω7 2OH	—	—	—	—	1.2
C _{16:1} ω5 2OH	—	—	—	—	16.2
C _{16:0} 2OH	—	—	—	—	25.7
C _{17:1} ω6 2OH	—	—	—	—	0.4
C _{18:1} 3OH	21.7	5.1	3.5	nd	—
C _{18:0} 3OH	53.4	5.7	7.6	nd	—
C _{20:1} 3OH	—	5.4	—	nd	—

^aFor symbols see standard definitions; nd, not determined.^bData from Sittig and Hirsch (1992).

the enrichment was incubated for 12 d in the dark at 20–25°C. Subsamples were then streaked onto medium PYGV¹ (Staley, 1968). Single colonies were further purified on the same medium.

MAINTENANCE PROCEDURES

The strain can be kept on slants at 4–5°C; it should be subcultured every three months. The strain is easily revived from lyophilized cultures.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Light microscopy with phase contrast and agar-coated glass slides (Pfennig and Wagener, 1986).

DIFFERENTIATION OF THE GENUS *GEMMOBACTER* FROM OTHER GENERA

Table BXII.α.53 lists the major features that differentiate *Gemmobacter aquatilis* from closely related taxa.

List of species of the genus *Gemmobacter*

- Gemmobacter aquatilis*** Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1988, 328^{VP} (Effective publication: Rothe, Fischer, Hirsch, Sittig and Stackebrandt 1987, 98.)
a.qua'ti.lis. L. neut. adj. *aquatilis* belonging to the water, describing its biotope.

Description as for the genus. Further characteristics are given in Tables BXII.α.53 and BXII.α.54.

The mol% G + C of the DNA is: 63.4 (*T_m*).

Type strain: ATCC 49971, DSM 3857, IFAM 1031.

TABLE BXII.α.54. Further characteristics of *Gemmobacter aquatilis*^a

Characteristic	Result/Reaction
<i>Carbon source utilization</i> (1 g/l):	
Glucose	+
Maltose	+
Lactose	+
Sucrose	+
Mannitol	+
Methanol (0.5% v/v)	—
Ethanol (0.4% v/v)	+
L-Arginine	+
L-Glutamate	+
Malate	+
Succinate	+

(continued)

1. PYGV consists of (per liter distilled water): peptone, 0.25 g; yeast extract, 0.25 g; glucose, 0.25 g; Hutner's basal salts medium (Cohen-Bazire et al., 1957), 20 ml; vitamin solution no. 6 (Staley, 1968), 10 ml.

TABLE BXII.α.54. (cont.)

Characteristic	Result/Reaction
Tartrate	+
N-Acetylglucosamine	—
Fermentation of (2 g/l):	
Glucose	+
Maltose	+
Lactose	+
Sucrose	+
Mannitol	+
Ethanol	+
Utilization of nitrogen sources (0.5 g/l):	
Ammonia	+
Nitrate	+
Urea (4.6 g/l)	—
Hydrolysis of (2.5 g/l):	
Casein	—
Gelatin	—

(continued)

TABLE BXII.α.54. (cont.)

Characteristic	Result/Reaction
Starch (3 g/l)	—
Reaction in litmus milk	—
Catalase	+
Cytochrome oxidase	+
Peroxidase	+
Major fatty acids:	
C _{18:1} ω7	+
C _{18:2} ω7, 13	+
C _{19:0} cyclo 7-8	+
Phospholipids:	
Phosphatidylglycerol	+
Phosphatidylethanolamine	—
Phosphatidylmonomethylethanolamine	—
Phosphatidyl dimethylethanolamine	+
Phosphatidylcholine	—
Biphosphatidylglycerol	+

^aFor symbols see standard definitions.

Genus VI. *Hirschia* Schlesner, Bartels, Sittig, Dorsch and Stackebrandt 1990, 449^{VP}

HEINZ SCHLESNER

Hirsch' i.a. M.L. fem. n. *Hirschia* honoring Peter Hirsch, a German microbiologist, who is an expert on budding and hyphal bacteria.

Rod-shaped or oval cells, 0.5–1.0 × 0.5–6.0 µm with one or two polar hyphae (prosthecae) with a diameter of 0.2 µm. Buds are formed at the tips of the hyphae. Daughter cells are motile by a single polar flagellum. **Gram negative. Aerobic and chemoor-ganotrophic. PHB is not stored.** Utilize various sugars and organic acids. **C₁-compounds are not used as a carbon source.** Am-monia and amino acids are nitrogen sources for growth. The ubiquinone system is a **Q-10** system. Hydroxy fatty acids are of the **3-OH** type.

The mol% G + C of the DNA is: 45–47 (*T_m*).

Type species: ***Hirschia baltica*** Schlesner, Bartels, Sittig, Dorsch and Stackebrandt 1990, 449.

FURTHER DESCRIPTIVE INFORMATION

Cells of *Hirschia* spp. are morphologically very similar to members of the genera *Hyphomicrobium* and *Hyphomonas* (Fig. BXII.α.73). Even the life cycle resembles that of the above genera, i.e., a motile swarmer cell loses the flagellum, the cell produces a hypha at one pole and a bud is formed at the tip of the hypha. A flagellum is produced at the distal pole of the bud. The mature bud separates from the mother cell by fission of the hyphal tip. Additional daughter cells may be produced by the mother cell.

Analysis of the 16S rRNA gene sequence of the type strain indicated that the genus *Hirschia* is a member of the *Alphaproteobacteria* and is distinctly but remotely related to members of the genera *Hyphomicrobium*, *Filomicrobium*, *Pedomicrobium*, and *Dichotomicrobium*. The 23S rRNA is not nicked (Schlesner et al., 1990).

ENRICHMENT AND ISOLATION PROCEDURES

The strains were obtained from brackish surface water (Kiel Fjord) after either enrichment or direct plating of sample on medium 13 (M13): peptone, 0.25 g/l; yeast extract, 0.25 g/l; glucose, 0.25 g/l; artificial seawater, 250 ml/l; vitamin solution, 10 ml/l; Hutners basal salts medium, 20 ml/l; 100 mM Tris/HCl (pH 7.5), 50 ml/l; agar, 18 g/l (Schlesner, 1986).

Three enrichment procedures are described by Schlesner et al., 1990:

1. Add glucose (0.05% final concentration) to the sample and incubate in a cotton stoppered Erlenmeyer flask at room temperature.
2. Add N-acetylglucosamine (0.1% final concentration) to the sample and incubate in a cotton-stoppered Erlenmeyer flask at room temperature.
3. The Petri dish procedure: A layer (about 1 cm) of water agar (1.8% agar in distilled water) on the bottom of a Petri dish (diameter, 25 cm) is covered with the sample to a depth of about 2 cm. The Petri dish is incubated at room temperature.

The cultures are examined by phase-contrast microscopy for the presence of hyphal bacteria. Samples are then streaked on M13 agar. After three weeks of incubation at 25°C, colonies are screened by phase-contrast microscopy. Pure cultures are obtained by repeatedly streaking on M13 agar.

MAINTENANCE PROCEDURES

Hirschia strains grown on slants can be kept at 4–5°C. They should be subcultured every three months. They are easily revived from lyophilized cultures and can be stored at –70°C in a solution of 50% glycerol in M13.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

This genus is easily distinguished from other hyphal bacteria by the yellow colonies formed on M13 agar.

DIFFERENTIATION OF THE GENUS *HIRSCHIA* FROM OTHER GENERA

Table BXII.α.55 lists the major features that differentiate *Hirschia* from other genera of budding prosthecate bacteria.

FURTHER READING

Schlesner, H., C. Bartels, M. Sittig, M. Dorsch and E. Stackebrandt. 1990. Taxonomic and phylogenetic studies on a new taxon of budding, hyphal *Proteobacteria*, *Hirschia baltica* gen. nov., sp. nov. Int. J. Syst. Bacteriol. 40: 443–451.

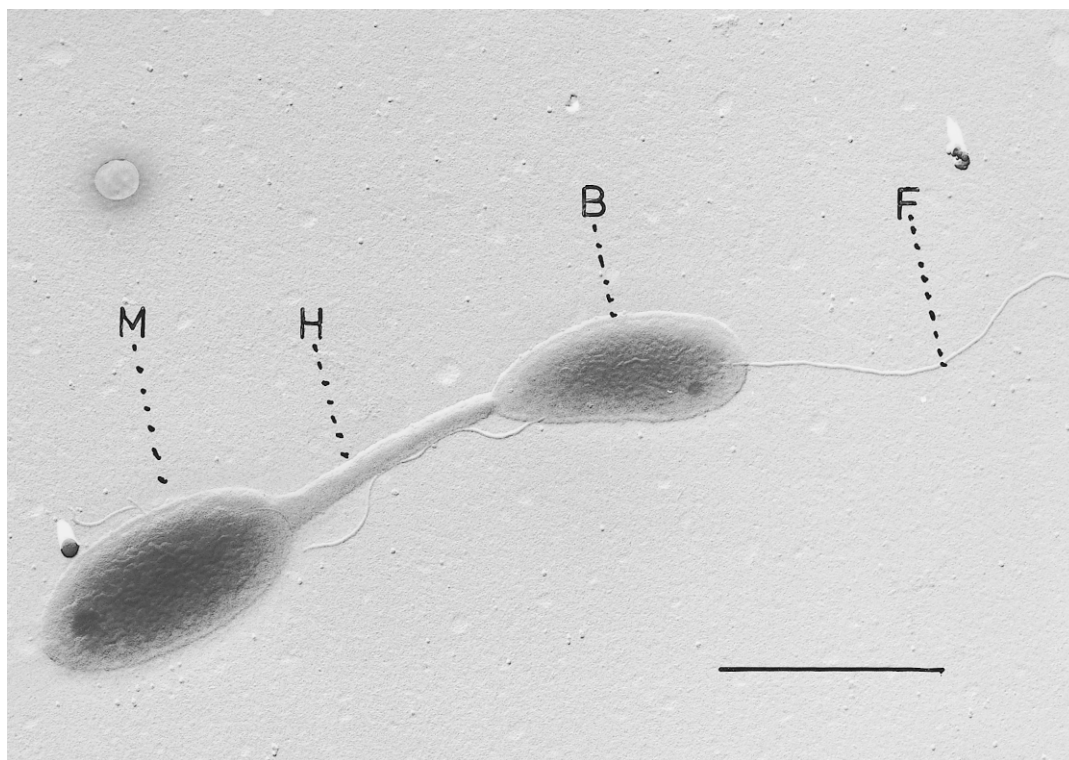


FIGURE BXII.α.73. *Hirschia baltica*, IFAM 1415, showing a mother cell (*M*), a hypha (*H*), and a bud (*B*) with polarly inserted flagellum (*F*). Platinum shadowing. Bar = 1 μ m.

List of species of the genus Hirschia

1. ***Hirschia baltica*** Schlesner, Bartels, Sittig, Dorsch and Stackebrandt 1990, 449

bal' ti.ca. M.L. fem. adj. *baltica* pertaining to the Baltic Sea.

Cells are rods, $0.5\text{--}1.0 \times 0.5\text{--}6.0$ μ m, elliptical or ovoid. Colonies are mucoid or dry and yellow. Optimal growth occurs between 22 and 28°C. Seawater is required for growth. Further characteristics are listed in Table BXII.α.56.

The habitat is brackish water. The isolates were obtained from the Baltic Sea (Kiel Fjord).

The mol% *G* + *C* of the DNA is: 45–47 (T_m); type strain 46 (T_m).

Type strain: SH 149, ATCC 49814, DSM 5838, IFAM 1418.

GenBank accession number (16S rRNA): X52909.

Additional Remarks: The 16S rRNA gene sequence of *Hirschia baltica* IFAM 1418 has been deposited at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany.

TABLE BXII.α.55. Differentiation of *Hirschia* from other hyphal budding bacteria

Characteristic	<i>Hirschia</i> ^a	<i>Hyphomicrobium</i> ^{b,c}	<i>Hyphomonas</i> ^{c,d}	<i>Filomicrobium</i> ^{e,e}	<i>Dichotomicrobium</i> ^{e,f}	<i>Pedomicrobium</i> ^{e,g}	<i>Rhodomicrobium</i> ^{h,i}
Cells ovoid, rod-, pear-, or bean-shaped	+	+	+	—	—	+	+
Cells nearly spherical	+	—	+	—	—	+	+
Cells nearly tetrahedral	—	—	—	—	+	—	—
Cells fusiform	—	—	—	+	—	—	—
Bud elongates with long axis of hyphae	+	+	+	+	+	—	+
Bud elongates perpendicular to hyphal long axis	—	—	—	—	—	+	—
Photosynthetic	—	—	—	—	—	—	+
Color of colonies ^j	Y	b	C or G	r	Rb	B	R

(continued)

TABLE BXII.α.55. (cont.)

Characteristic	<i>Hirschia</i> ^a	<i>Hyphomicrobium</i> ^{b,c}	<i>Hyphomonas</i> ^{c,d}	<i>Filomicrobium</i> ^{c,e}	<i>Dichotomicrobium</i> ^{c,f}	<i>Pedomicrobium</i> ^{c,g}	<i>Rhodomicrobium</i> ^{h,i}
Able to use C ₁ -compounds as the sole source of carbon	—	+	—	—	—	—	—
Able to use amino acids as carbon source	+	—	+	—	—	—	—
Able to use sugars as carbon source	+	—	—	—	d	d	—
Able to use organic acids as carbon source	+	+	—	+	+	+	+
Fe and/or Mn are oxidized	—	v	—	—	—	+	—
Number of hyphae	1 or 2	1 or 2	1 or 2	2 or 3	up to 4	1 to 5	1 or 2
Flagellation monotrichous	+	+	+	—	—	+	—
Flagellation peritrichous	—	—	—	—	—	—	+
Main quinone component:							
Q9		+		+			
Q10	+		+		+	+	+
Q11			+				
Mol% G + C of DNA (<i>T_m</i>)	45–47	59–65	57–62	62	62–64	62–67	62–64

^aData from Schlesner et al. (1990).

^bData from Hirsch (1989).

^cData from Sittig and Hirsch (1992).

^dData from Weiner et al. (1985).

^eData from Schlesner (1987).

^fData from Hirsch and Hoffmann (1989a).

^gData from Gebers (1981).

^hData from Duchow and Douglas (1949).

ⁱData from Collins and Jones (1981).

^jFor symbols see standard definitions. Y, yellow; B, dark brown; b, brown; C, colorless; G, gray; R, red; r, light red; Rb, reddish brown.

Other Organisms

Besides the three strains of *H. baltica* (IFAM 1408, 1415, and 1418^T), a fourth strain (IFAM 1538) was isolated and investigated. Morphological characteristics are similar to *H. baltica*, the base composition of the DNA is 47 mol% G + C (*T_m*). Differences are found in the utilization of carbon sources, i.e., the ability to utilize lactose and glycerol and the inability to utilize acetate,

propionate, pyruvate, lactate, or alanine. Furthermore, with the LRA ZYM Osidase System (API bioMérieux, Montalieu, France) the enzymes α-D-galactosidase, β-D-galactosidase, phospho-β-D-galactosidase, β-D-fucosidase, and β-D-lactosidase were found in IFAM 1538, but not in the strains of *H. baltica*, while only the strains of *H. baltica* tested positive for α-D-arabinosidase.

TABLE BXII.α.56. Characteristics of *Hirschia baltica*^a

Characteristic	Reaction
Carbon source utilization:	
Cellobiose	+
Fructose	d
Glucose	+
Maltose	d
Lactose	—
Xylose	—
Raffinose	—
Rhamnose	d
Ribose	—
Trehalose	—
Adonitol	—

(continued)

TABLE BXII.α.56. (cont.)

Characteristic	Reaction
Ethanol	—
Glycerol	—
Mannitol	—
Methanol	—
Acetate	+
Adipate	—
Butyrate	+
Caproate	+
Citrate	—
Formate	—
Fumarate	—
Lactate	+
Malate	—

(continued)

TABLE BXII.α.56. (cont.)

Characteristic	Reaction
Propionate	+
Pyruvate	+
Succinate	d
Gluconate	+
Glucuronate	d
Alanine	+
Arginine	d
Asparagine	+
Aspartic acid	+
Glutamic acid	+
Glutamine	+
Glycine	—
Histidine	—
Isoleucine	+
Leucine	d
Lysine	—
Proline	+
Serine	d
Valine	—
Methylammonium chloride	—
Formamide	—
N-Acetylglucosamine	d
Amygdaline	d
<i>Utilization of nitrogen sources:</i>	
Ammonia	+
Glutamic acid	+
Nitrate	+
Urea	+
Acetamide	—
N-Acetylglucosamine	—
Formamide	—

(continued)

TABLE BXII.α.56. (cont.)

Characteristic	Reaction
Nicotinic acid	—
<i>Hydrolysis of:</i>	
Alginates	+
DNA	d
Gelatin	+
Starch	d
Tween 80	+
Production of NH ₃ from peptone	+
Production of H ₂ S from thiosulfate	d
<i>Sensitive to:</i>	
Ampicillin	+
Oxytetracycline	+
Polymyxin B	+
Streptomycin	+
Tetracycline	+
Bacitracin	—
Nalidixic acid	—
Oxacillin	—
<i>Fatty acids:</i>	
C _{16:0}	+
C _{18:1 ω7}	+
C _{18:2 ω7, 13}	+
Cyclopropane fatty acids	(+) ^b
Branched fatty acids	—
<i>Phospholipids:</i>	
Phosphatidylglycerol	+
Glycolipids	—
Mesodiaminopimelic acid	+

^aFor symbols see standard definitions.^bPresent in minor amounts (up to 10% of the total fatty acids).

Genus VII. *Hyphomonas* (ex Pongratz 1957) Moore, Weiner and Gebers 1984, 71^{VP} emend. Weiner, Melick, O'Neill and Quintero 2000, 466

RONALD M. WEINER

Hy.pho.mo' nas. Gr. n. *hyphos* filament; Gr. n. *monas* a unit, monad; M.L. fem. n. *Hyphomonas* hypha-bearing unit.

Rod-shaped to oval mature cells measure 0.5–1.0 × 1.0–3.0 µm and may become larger and rounder just prior to bud formation. Unicellular. Cell division occurs by budding. **Buds are produced at tips of single polar prosthecae**, which are 0.2–0.3 µm in diameter and 1–5 times the length of the cell body. Prosthecae are nonseptate and rarely branch under normal growth conditions. Pleomorphic. *Hyphomonas* spp. have a **biphasic life cycle** and normally generate only a single polar prostheca (hypha). Young daughter cells (i.e., newly formed buds) are oval to pear shaped, lack prosthecae, and are smaller than the mother cell. Motile by means of a **single polar to lateral flagellum located on developing buds** or young daughter cells. **Gram negative**. Not acid-fast. Aerobic. Nonsporeforming. Chemooorganotrophic. *Hyphomonas* spp. **catabolize amino acids or tricarboxylic acid cycle intermediates** for energy and growth. All strains investigated thus far are catalase positive, oxidase positive, nonproteolytic, nonsaccharolytic, and nonpathogenic. Gelatin and starch not hydrolyzed; no indole from tryptophan; no DNase, ornithine decarboxylase, lysine decarboxylase, or coagulase activity. With one exception, all species denitrify. Amino acids are required for heterotrophic growth. Optimal temperature for growth ranges from 22 to 37°C at one atmosphere of pressure. Prefer slightly alkaline conditions for growth. **All strains were isolated from marine sources.**

The mol% G + C of the DNA is: 57–64.

Type species: ***Hyphomonas polymorpha*** (ex Pongratz 1957) Moore, Weiner and Gebers 1984, 71.

FURTHER DESCRIPTIVE INFORMATION

Normal-appearing cells of the type strain of *Hyphomonas polymorpha* (ATCC 33881, IFAM PS 728) are shown in Fig. BXII.α.74. All species include strains that have capsules and/or holdfasts (Langille and Weiner, 1998; Quintero, et al., 1998). Old cultures or poor growth conditions produce a large number of aberrant cell forms. These include giant cells, spindle-shaped or triangular cells, cells with unusually long or branched prosthecae, cells with intercalary buds and cells with hyphae originating from locations other than the poles. Daughter cells may be half their normal dimensions. Polyphosphate and poly-β-hydroxybutyrate granules, which are normally present (Weiner et al., 1985), may become especially pronounced under poor growth conditions. A number of traits set *Hyphomonas* spp. apart from other bacteria that bud through a prostheca (Table BXII.α.57).

The appearance of the cell wall in thin sections is typical of other Gram-negative bacteria. The cytoplasmic membrane is continuous with the cell wall, and no organized membrane structures are observed. The composition of the cell wall of *Hyphomonas*

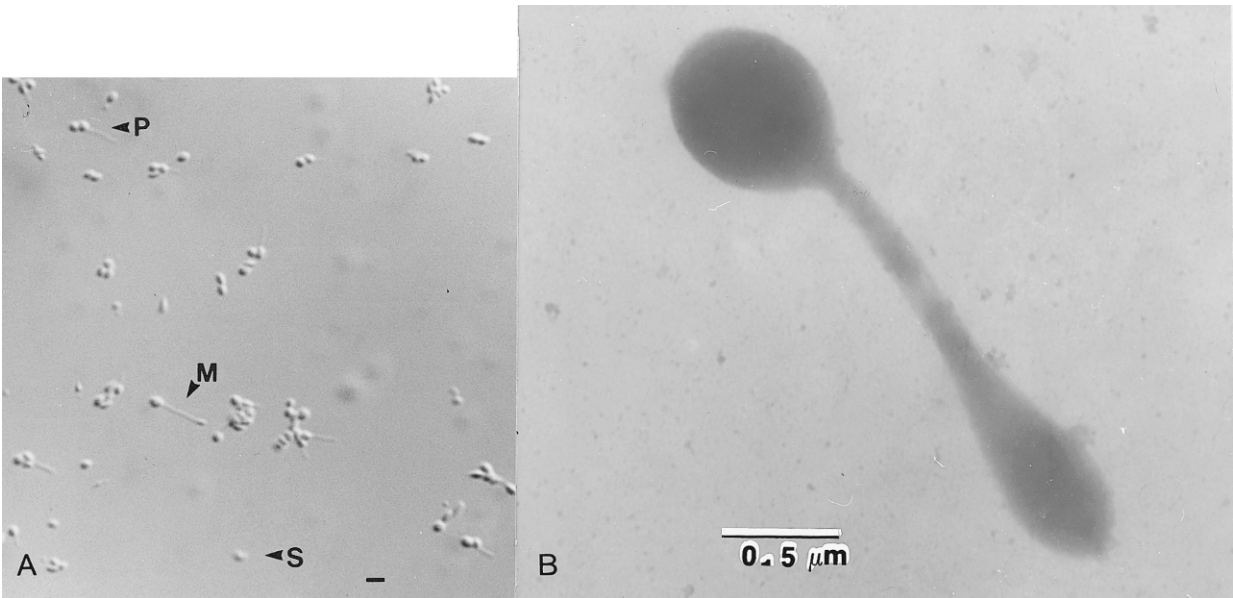


FIGURE BXII.α.74. *H. polymorpha*, type species. A, type strain S (ATCC 33881; IFAM PS 728). Nomarski photomicrograph showing budding mother cell (M), cell with prostheca (P), and swarmer cells (S). Flagella are not visible. Bar = 1 μm. B, strain R (ATCC 33880; IFAM PR 727). Scanning electron micrograph of mother cell showing bud formation. (Courtesy of Sorando and R. Weiner).

TABLE BXII.α.57. Differential characteristics of the genera of bacteria that reproduce by budding from the tip of the prostheca ^a

Characteristic	<i>Hyphomonas</i>	<i>Hirschia</i> ^b	<i>Hyphomicrobium</i>	<i>Pedomicrobium</i>	<i>Rhodomicrobium</i>
Photosynthetic	–	–	–	–	+
Able to use C ₁ compounds as a source of carbon	–	–	+	D	–
Requires amino acids as a source of carbon	+	–	–	–	–
Able to use a variety of organic acids and alcohols as a source of carbon	–	–	–	+	+
Produces spores or cysts	–	–	–	–	+
Capable of depositing a heavy layer of iron or manganese salts on the cell surface	–	–	–	+	–
Main cell body of mother cell retains size and shape during cell cycle	–	–	+	–	+
Number of hyphae	1 ^c	1–2	1 ^c	1–5	2
Position of hyphae	Polar	Polar	Polar		Polar
PHB storage granules	+	–	+	+	+
Colony color	G/b	Y	b	B	R
Mol% G + C	57–64	45–47	59–65	62–67	62–64

^aSymbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, differs, depending on species of genus; G, gray; b, brown; B, dark brown; R, red; Y, yellow (Moore et al., 1984).

^bSchlesner et al., 1990.

^cOccasionally two hyphae are present.

neptunium was found to be much more similar to that of *Escherichia coli* than to a strain of *Hyphomicrobium* (Jones and Hirsch, 1968). The cells are sensitive to detergents (see Table BXII.α.58) and are readily lysed in a solution of ethylenediaminetetraacetate with 1% sodium lauryl sulfate. Cells stain well with aniline dyes. The use of Lugol’s iodine as a mordant facilitates the staining of the narrow hyphae. Best results are obtained with silver impregnation.

The life cycle of these organisms is generally similar to those of other prosthecate, budding bacteria (Hirsch, 1974a) and has been investigated in some detail (Wali et al., 1980; Moore, 1981a; Quintero, et al., 1998; Weiner, 1998). At 36°C, *Hyphomonas nep-*

tunium requires about 265 min to complete a full cycle. During the first 85 min, the cell exists as a motile swarmer cell. The flagellum becomes detached, and the cell produces a prostheca at one, usually the narrower, of the cell poles. Some species produce polar fimbriae at the other pole (Quintero et al., 1998). The major outer membrane protein profile undergoes a marked change (Shen et al., 1989). This prosthecal growth stage requires approximately 95 min of the total cycle. At the end of this stage, the size of the main cell body may increase by up to 2–4 times. The bud development stage occupies the remaining 85 min of the life cycle. The bud is produced at the very tip of the prostheca and synthesizes a single flagellum (Quintero et al., 1998). The

TABLE BXII.α.58. Characteristics of species of the genus *Hyphomonas*^a

Characteristic	<i>H.</i> <i>polymorpha</i> PS 728/PR 727 ^T	<i>H.</i> <i>adhaerens</i> MHS-3	<i>H.</i> <i>hirschiana</i> VP5 ^T	<i>H.</i> <i>jannaschiana</i>		<i>H.</i> <i>johnsonii</i> MHS-2	<i>H.</i> <i>neptunium</i> LE617 ^T /H13	<i>H.</i> <i>oceanitis</i> SCH 1325 ^T	<i>H.</i> <i>rosenbergii</i> VP-6
				VP2 ^T /VP4	VP1/VP3				
<i>Type of growth in broth:</i>									
Granular		+			+	+			+
Pellicle			+						
Ropy		+		+					+
Turbid	+						+	+	
Optimal temperature (°C)	30–37	25–37	25–31	37	37	25–37	30–37	20–30	25–45
% NaCl for growth, optimal	0.5–3.5	3.5–7.5	3.5–7.5	3.5–7.5	3.5–7.5	2.5–5.0	2.5–5.0	2.5–5.0	3.0–5.0
% NaCl for growth, range	0.5–5.0	1.5–12.0	2.0–15.0	2.0–5.0	2.0–15.0	1.5–6.0	1.0–7.5	1.0–7.5	1.0–12.0
Optimal pH	7.0–7.4	7.6	7.6	7.6	7.6	7.4	8.0	7.6	8.0
Microaerophilic growth	–	Slight	–	Slight	Slight	Slight	–	Slight	–
Requires methionine	+ ^b	–	+	–	–	–	+	+	nd
Requires biotin	–	–	–	+	+	–	–	+	nd
Film formation on growth vessel	– or slight ^c	+++	Slight	Slight	+	±	Slight	–	++
Rosette formation	–	–	–	–	+	+	–	–	+
Brown pigment at 31–41°C	–	±	–	+	+	–	–	–	+
Resistance to mechanical lysis ^d	2–5	nd	9	9	9	nd	2	6–8	nd
Hemolysis on sheep blood agar	χ	χ	χ	α	α	α	α	χ	α
Growth on TSI agar	–	–	–	–	–	–	–	–	–
Growth on MacConkey agar	–	–	–	–	–	–	–	–	–
Mannitol fermentation	–	–	–	–	–	–	–	–	–
Acid from glucose, lactose	–	–	–	–	–	+	–	–	–
Gelatin hydrolysis	–	–	–	–	–	–	–	–	–
Starch hydrolysis	–	–	–	–	–	–	–	–	–
Indole from tryptophan	–	–	–	–	–	–	–	–	–
NO ₃ [–] → NO ₂ [–]	–	+	+	+	+	+	+	+	+
NO ₂ [–] → N ₂ , NH ₃	–	+	+	+	+	+	+	+	+
DNase	–	+	–	–	–	–	–	–	+
Ornithine decarboxylase	–	–	–	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+
Coagulase	–	–	–	–	–	–	–	–	–
<i>Sensitivity to:</i>									
Tellurite (0.5%)	S	S	S	S	S	S	S	S	S
Crystal violet (0.2%)	S	S	S	S	S	S	S	S	R
Neutral red (0.4%)	R	S	R	R	R	R	R	R	R
Brilliant green (0.2%)	S	S	S	S	S	S	S	S	S
Methylene blue (10%)	S	S	S	S	S	R	S	S	S
Sodium lauryl sulfate (1%)	S	S	S	S	S	S	S	S	S
Teepol 610 (80%)	S	nd	S	S	S	nd	S	S	nd
Methyl violet (0.04%)	R	S	R	R	R	R	R	R	S
Pyronin (0.4%)	S	nd	S	S	S	nd	S	S	nd
NP-40 (0.01%)	I	nd	S	S	S	nd	S	S	nd
Tween 80 (0.01–1.0%)	R	R	R	R	R	R	R	S	R
Erythromycin (15 µg)	S	nd	S	S	S	nd	S	S	nd
Rifampin (5 µg)	S	nd	S	S	S	nd	S	S	nd
Cephalothin (30 µg)	S	nd	S	S	S	nd	S	S	nd

(continued)

TABLE BXII.α.58. (cont.)

Characteristic	<i>H. polymorpha</i> PS 728/PR 727 ^T	<i>H. adhaerens</i> MHS-3	<i>H. hirschiana</i> VP5 ^T	<i>H. jannaschiana</i> VP2 ^T /VP4 VP1/VP3		<i>H. johnsonii</i> MHS-2	<i>H. neptunium</i> LE617 ^T /H13	<i>H. oceanitis</i> SCH 1325 ^T	<i>H. rosenbergii</i> VP-6
Novobiocin (30 µg)	S	S	S	S	S	S	S	R	S
Kanamycin (30 µg)	S	S	S	S	S	S	S	S	S
Chloramphenicol (30 µg)	S	S	S	S	S	S	S	S	S
Penicillin (10 IU) ^c	S	R	I	S	S	S	I	R	S
Ampicillin (10 µg)	S	R	S	S	S	S	S	R	R
Streptomycin (10 µg)	I	S	S	S	S	S	S	I	S
Mol% G + C of DNA	60–61	60	57	60	60	64	62–60	59	61

^aSymbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; S, sensitive; I, intermediate; R, resistant; nd, not determined. Data from Weiner et al. (1985) and Weiner et al. (2000).

^b*H. polymorpha* PR 727^T does not require methionine, but growth is stimulated by methionine.

^c+ / –, patchy film; ++, contiguous, clearly visible film; + + +, thick, uniform film.

^dMinutes required for 99% lysis by Brownill Biosonik IV sonicator; low probe at full power.

^eSensitivities were assessed on 50% concentrations of marine broth (Zobell, 1941). Sensitivity on other medium may be different (Hirsch, 1974a).

mother cell is pushed through the medium by the motile bud, in contrast to *Hyphomicrobium* species, which are pulled by the motile bud (Moore, 1981a). Whether this constitutes a serious diagnostic feature, however, is uncertain. The mature bud eventually separates from the mother cell by fission of the prosthecal tip. After a short period of prosthecal growth, further daughter cells may be produced by the mother cell. Altering the temperature of growth not only influences the time course of the life cycle but also results in a shift in the relative proportion of the various cell types present in a given culture. DNA synthesis is discontinuous and occurs just prior to bud formation. Membrane structures are involved in DNA segregation (Zerfas et al., 1997).

Good growth occurs on Casamino acids, blood agar, and other rich media. The addition of sea salts is required by nearly all strains and is stimulatory to the others (Havenner et al., 1979; Weiner et al., 2000). Growth in a defined medium supplemented with a mixture of glutamic acid, aspartic acid, methionine, and serine as substrates has been reported for *Hyphomonas neptunium* (Havenner et al., 1979). *H. hirschiana* and *H. polymorpha* have the same requirements as *H. neptunium*; *H. jannaschiana* requires biotin but not methionine; *H. oceanitis* requires the four amino acids plus biotin, folic acid, pyridoxine, riboflavin, and *p*-aminobenzoic acid. *H. johnsonii* differs from the other species of *Hyphomonas*, being able to utilize glucose as a carbon source in the presence of amino acids. *H. adhaerens* does not have auxotrophic requirements for amino acids but requires them (or peptone or protein) for carbon and energy.

CO₂ is the primary end product of metabolism, and the medium becomes alkaline, probably due to the evolution of ammonia (Leifson, 1964; Havenner et al., 1979). Deamination and tricarboxylic acid cycle oxidation of amino acids is the major catabolic pathway (Havenner et al., 1979).

No growth on mineral salts media with CaCO₃, acetate, propionate, lactate, ethanol, glycerol, glycine, or C₁ compounds as the sole source of carbon. Growth is not influenced by light.

Under certain conditions, some strains may produce a dark brown, acid-insoluble, base-soluble pigment (see Table BXII.α.58), identified as a pyromelanin, the product of *p*-hydroxyphenylpyruvate hydroxylase via homogentisic acid (Kotob et al., 1995).

H. neptunium, *H. polymorpha*, *H. oceanitis*, and *H. hirschiana* have a Q-11 ubiquinone type along with a significant amount of Q-10 (approximately 10% of total ubiquinones) and minor amounts of Q-9 and Q-12. *H. jannaschiana* has Q-10 as its major quinone with trace amounts of Q-9 and Q-11 (Urakami and Komagata, 1987b).

Hyphomonas spp. synthesize high percentages of octadecenoic acid, (C_{18:1}; Table BXII.α.59), albeit in varying amounts. These range from 11% in *H. jannaschiana* to 80% in MHS-3, a value exceeding that found in all extant *Hyphomonas* spp. but correlating well with most other budding/prosthecae bacteria (i.e., *Rhodomicrobium*, *Pedomicrobium*, and *Hyphomicrobium*) (Urakami and Komagata, 1987b; Sittig and Hirsch, 1992). *Hyphomonas* also contains novel lipids (Batrakov et al., 1996).

The membrane protein profile similarities of the eight species range from 30 to 80% (Dagasan and Weiner, 1986; Weiner et al., 2000), and all but one species, *H. johnsonii*, synthesize large amounts of a 47 kDa protein. All species are serologically related, based upon exposed determinants (Weiner et al., 2000).

Proteases (Shi et al., 1997) and S-layer proteins (Shen and Weiner, 1998) have been identified and characterized.

Table BXII.α.60 shows the % overall DNA–DNA homologies among the eight extant *Hyphomonas* species.

ENRICHMENT AND ISOLATION PROCEDURES

Most of the currently available isolates have been obtained by direct plating of samples onto commonly used media such as blood agar, medium 383 (casitone, 2 g/l; yeast extract, 1 g/l; MgCl₂, 1 g/l; [Leifson, 1964]) and marine agar (Difco 2216; [Zobell, 1941]). However, a simple procedure has been reported for their isolation from water samples (Moore, 1981b). The sample is brought to 0.005% (w/v) peptone and 0.005% (w/v) yeast extract. The cultures are incubated aerobically, and after times ranging from about one day to a week or more, are plated onto solid medium. The colonies, which arise relatively slowly, are screened microscopically for cells with the typical morphology of *Hyphomonas* species and are replated for purification and identification. Three of the eight species have been isolated from warm water hydrothermal vents, either from shellfish beds near the Galapagos Islands, at a depth of 2600 m (Jannasch and Wir-

TABLE BXII.α.59. Fatty acid profiles of *Hyphomonas*^a

Strain	Reference	% Total fatty acids ^b						
		C _{15:0}	C _{16:0}	C _{16:1}	C _{17:0}	C _{17:0 cyclo}	C _{17:1}	C _{18:1}
<i>H. polymorpha</i> PS728 ^T	Sittig and Hirsch, 1992 ^c	9	0	1	25	nd ^d	14	12
	Weiner et al., 2000 ^e	6	2	1	23	nd	24	15
	Urakami and Komagata, 1987b ^f	10	2	2	19	44	nd	10
<i>H. adhaerens</i> MHS-3 ^T	Weiner et al., 2000 ^e	0	10	2	0	nd	0	80
<i>H. hirschiana</i> VP-5 ^T	Sittig and Hirsch, 1992 ^c	0	30	2	0	nd	0	54
	Weiner et al., 2000 ^e	7	9	3	11	nd	13	39
	Urakami and Komagata, 1987b ^f	1	9	7	2	11	nd	67
<i>H. jannaschiana</i> VP-2 ^T	Sittig and Hirsch, 1992 ^c	5	4	1	18	nd	14	22
	Weiner et al., 2000 ^e	5	2	0	31	nd	22	11
	Urakami and Komagata, 1987b ^f	1	7.2	3	6	10	nd	65
<i>H. johnsonii</i> MHS-2 ^T	Weiner et al., 2000 ^e	0	21	3	0	nd	0	64
<i>H. neptunium</i> Le670 ^T	Sittig and Hirsch, 1992 ^c	10	5	1	19	nd	18	19
	Weiner et al., 2000 ^e	8	8	4	10	nd	14	37
	Urakami and Komagata, 1987b ^f	8	2	2	16	55	nd	10
<i>H. oceanitis</i> SCH89 ^T	Sittig and Hirsch, 1992 ^c	0	34	6	0	nd	0	53
	Weiner et al., 2000 ^e	0	27	6	0	nd	0	49
	Urakami and Komagata, 1987b ^f	0	22	13	1	1	nd	60
<i>H. rosenbergii</i> VP-6 ^T	Weiner et al., 2000 ^e	2	32	6	0	nd	3	43

^aData from Weiner et al., 2000.^bRounded to nearest 1% of total detected fatty acids.^cCells harvested in late logarithmic phase at 25°C.^dnd; no data.^eCells harvested in late logarithmic phase at 27°C.^fCells harvested during stationary phase at 30°C.TABLE BXII.α.60. DNA–DNA reassociation values of the eight *Hyphomonas* species^a

Strains	<i>H. polymorpha</i> PS-728 ^T	<i>H. adhaerens</i> MHS-3 ^T	<i>H. neptunium</i> LE-670 ^T	<i>H. johnsonii</i> MHS-2 ^T	<i>H. hirschiana</i> VP-5 ^T	<i>H. oceanitis</i> Sch89 ^T	<i>H. jannaschiana</i> VP-1	<i>H. rosenbergii</i> VP-6 ^T
PS-728	100							
MHS-3	32	100						
LE-670	27	66	100					
MHS-2	13	25	25	100				
VP-5	34	19	54	12	100			
Sch89	28	11	12	23	20	100		
VP-1	8	41	32	18	23	26	100	
VP-6	5	30	11	21	19	2	4	100

^aResults calculated as the relationship of at least three separate experiments, averaging six values including the reciprocal. All the values listed are combined averages from the reciprocal hybridizations, which are within 10% of one another. Data from Weiner et al., 2000.

sen, 1981), or from the Guyamas Basin at a depth of 2000 m. Members of the genus have been identified as primary colonizers of submerged surfaces in marine waters (Baier et al., 1983; Fro-lund et al., 1996). Due to their relatively slow growth, they are not readily isolated, though they can be well represented on submerged surfaces in marine waters (Weidner et al., 1996).

MAINTENANCE PROCEDURES

The members of this genus are generally quite hardy and will survive up to several months in liquid or solid growth medium at 4°C. They are easily revived from lyophilized cultures and can be stored at –20°C over a period of several years, or for a longer period of time at –70°C, in a solution of 60% glycerol in 0.05 M KH₂PO₄ at pH 7.

DIFFERENTIATION OF THE GENUS *HYPHOMONAS* FROM OTHER GENERA

The eight species have signature 16S rDNA sequences (Weiner et al., 2000) that place them in the *Alphaproteobacteria* (Stacke-brandt et al., 1988a). Each is related to the other above the 96% similarity level. A member of the most closely related genus, *Hirschia*, (Schlesner et al., 1990), is related to each of the strains

of *Hyphomonas* at the 90 ± 1% level. 16S rDNA sequence analyses support the notion that *Hyphomonas*, a genus of marine bacteria, is more closely related to the marine line of *Caulobacter* (Stahl et al., 1992) than to terrestrial, budding, prosthecate genera (e.g., *Hyphomicrobium*; Fig. BXII.α.75).

Table BXII.α.57 lists other major features which differentiate *Hyphomonas* from other genera of prosthecate, budding bacteria. Support for the separation of this genus is also indicated by the low level of intergeneric cross-reactions seen in serological studies (Powell et al., 1980; Weiner et al., 2000), DNA–DNA homology studies (Moore and Hirsch, 1972; Moore and Staley, 1976; Gebers et al., 1984; Weiner et al., 2000), rRNA–DNA studies (Moore, 1977), fatty acid analyses (Urakami and Komagata, 1987b; Sittig and Hirsch, 1992; Weiner et al., 2000), rRNA cistron similarities (Roggentin and Hirsch, 1989), comparisons of membrane protein profiles (Dagasan and Weiner, 1986; Weiner et al., 2000), and other criteria (Gebers et al., 1985; Köbel-Boelke et al., 1985; Nikitin et al., 1990).

ACKNOWLEDGMENTS

The authors thank J. Poindexter, R. Gebers, J. Smit, and H. Jannasch for providing isolates and helpful discussion. Colleagues and members of my

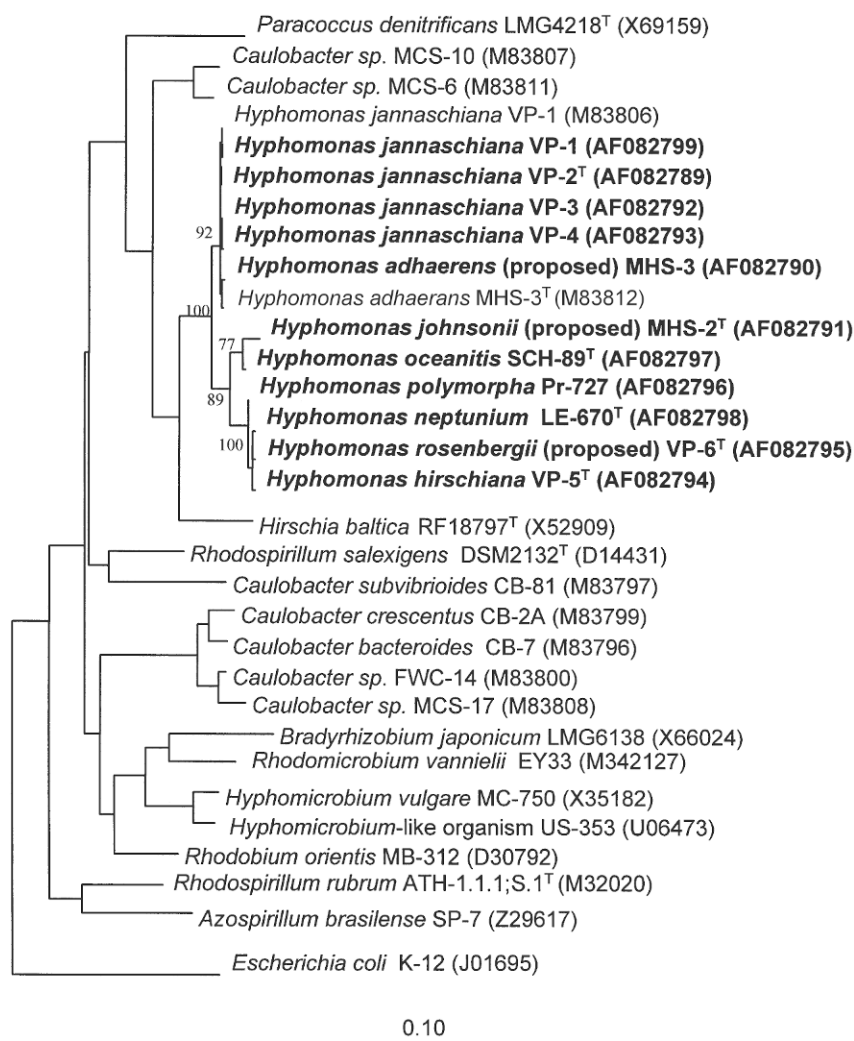


FIGURE BXII.α.75. Phylogenetic analysis of *Hyphomonas*. Tree constructed using the fastDNA maximum likelihood program from the Ribosomal RNA Database Project (RDP) (Larsen et al., 1993). Sequences obtained in this study are in boldface type. Sequences for comparison were obtained from the RDP (Larsen et al., 1993). Bootstrap values (expressed as percentages of 100 replications), pertinent to *Hyphomonas* species, are shown at the appropriate branch points. *Escherichia coli* MRE600 was used as an outgroup to establish the root of the tree. Data are reproduced with permission from R.M. Weiner et al., International Journal of Systematic and Evolutionary Microbiology: 50: 459–469, 2000, ©International Union of Microbiological Societies.

laboratory who have contributed to the manuscript are also acknowledged. These include K. Busch, R. Devine, L. Dagasan, R. Gherna, K. Guthrie, G. Lacy, J. Johnson, M. Melick, K. O'Neill, E. Quintero, F. Singleton, and H. G. Trüper. A portion of this work was supported by Maryland Industrial Partnerships (MIPS), FDA-JIFSAN, and Maryland Sea Grant.

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *HYPHOMONAS*

Most criteria, other than 16S rDNA sequence analysis, support the placement of *Hyphomonas* as a discrete genus but do not

clearly establish relationships between all species. These criteria include serological identity based upon surface antigen (Powell

et al., 1980; Weiner et al., 2000), some DNA–DNA homology results (Moore and Hirsch, 1972; Moore and Staley, 1976; Gebers et al., 1986, Weiner et al., 2000), rRNA–DNA studies (Moore, 1977), fatty acid analyses (Weiner et al., 2000), and comparisons of membrane protein profiles (Dagasan and Weiner, 1986; Weiner et al., 2000).

The best criterion to ascertain the interrelationship of *Hyphomonas* species is genetic similarity. The phylogenetic tree (Fig. BXII.α.75) shows the relatedness of each of the species of *Hyphomonas* according to 16S rDNA sequence analysis. *H. rosenbergii* (nov.), *H. hirschiana*, *H. polymorpha*, and *H. neptunium* cluster at the 99.4% similarity level. *H. adhaerens* (nov.) and *H. jannaschiana* share sequence similarities of 99.3% and, as expected, each of the strains of *H. jannaschiana* is related at a high level (99.3%) as well. *H. johnsonii* (nov.) is most closely related to *H. oceanitis* at the 98.7% level, and neither of these species is closely related to other species of *Hyphomonas*.

Thus, 16S rDNA sequence analyses divide the eight species of *Hyphomonas* into: 1) a tight group of four species, *H. rosenbergii*, *H. hirschiana*, *H. polymorpha*, and *H. neptunium*; 2) a group with two species, *H. adhaerens* and *H. jannaschiana*; and 3) a less tight group of two species, *H. johnsonii* and *H. oceanitis*. Likewise, the DNA–DNA hybridization data (Table BXII.α.60) show *H. neptunium* to be closely related to *H. hirschiana* and supports both of the two species groupings, i.e., *H. adhaerens* and *H. jannaschiana* plus *H. johnsonii* and *H. oceanitis*. (DNA–DNA hybridization data also suggest that *H. neptunium* forms a tighter relationship with *H. adhaerens* based upon DNA–DNA reassociation values (Table BXII.α.60) than by 16S rDNA sequence comparisons.

List of species of the genus *Hyphomonas*

1. ***Hyphomonas polymorpha*** (ex Pongratz 1957) Moore, Weiner and Gebers 1984, 71^{VP}
po.ly.mor'pha. Gr. adj. *poly* many; Gr. n. *morphe* shape, body; M.L. adj. *polymorpha* many shapes.

Pongratz (1957) reported that upon isolation on solid media, colonies appear as smooth or rough types. The smooth colonies are round, convex, watery, and translucent and can be emulsified easily. A capsule covers the main body of the reproductive cell (not prostheca). Rough colonies are rare. They are smaller and dry and form a central crater after several days. The colonies are not readily emulsified, and suspensions remain granular. They lack capsules.

No growth on inulin, dextrin, glycogen, esculin, glycerol, erythritol, mannitol, sorbitol, or urea. Reduces neutral red, methylene blue and Janus green. Produces H₂S. Other characteristics are given in Table BXII.α.58.

Contrary to the original description (Pongratz, 1957), NO₃⁻ and NO₂⁻ are not reduced, indole is not formed from tryptophan, and the rough strain, PR 727, is motile. It is not known whether these differences are due to the continuous subculturing of these strains.

Not virulent for mice (5 ml), rats (1 ml), guinea pigs (1 ml), or rabbits (1 ml) if a suspension which contains 5 × 10⁹ cells/ml is injected subcutaneously or intraperitoneally. The organisms cannot be recovered from treated animals. The major fatty acids are C_{17:0}, C_{17:1}, and octadecanoate (C_{18:1}) at 24, 19, and 14%, respectively, of the total fatty acids (Table BXII.α.59). Isolated only once from a patient (deep sea diver) with infectious sinusitis. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

However, DNA–DNA reassociation values can be inflated by shared plasmids and other horizontal genetic exchange).

Phenotypic characteristics tend to support these placements, as can be ascertained from an examination of Table BXII.α.58. Rosette formation is a result of holdfast (polar adhesive capsule) production. *H. rosenbergii* synthesizes both a capsule that surrounds the entire cell, including main body, prostheca and bud, and a holdfast (Langille and Weiner, 1998). *H. adhaerens* produces a capsule that surrounds only the main body of the mother cell, mediating side/side adhesion as well as pole/pole adhesion (Quintero and Weiner, 1995; Quintero et al., 1998). The holdfast of *H. rosenbergii* and capsule of *H. adhaerens* are chemically different, while sharing certain properties such as an abundance of amino sugars (Quintero and Weiner, 1995). The LPS of *H. adhaerens* is antigenically unique from that of any other surface antigen of any other *Hyphomonas* species (Weiner et al., 2000).

H. johnsonii is clearly the outlier species of *Hyphomonas* based on almost all criteria. So far, it is the only species shown to be able to utilize sugars for carbon and energy. Additionally, it has little DNA–DNA hybridization similarity with other *Hyphomonas* species (Table BXII.α.60) and lacks some genus-specific phenotypic characteristics (Jones and Krieg, 1984), including outer membrane proteins (Weiner et al., 2000). Furthermore, it has the lowest serological identity with any of the other *Hyphomonas* species (Weiner et al., 2000).

The site of isolation (e.g., hydrothermal vent, benthos, coastal) does not appear to have a bearing on the relationships between *Hyphomonas* species.

The mol% G + C of the DNA is: 60 (Bd) (Mandel et al., 1972); rough strain (IFAM PR 727, ATCC 33880), 61 (Bd).
Type strain: PS728, ATCC 33881; IFAM PS728.
GenBank accession number (16S rRNA): AJ227813.

2. ***Hyphomonas adhaerens*** Weiner, Melick, O' Neill and Quintero 2000, 467^{VP}
ad.hae'rens. L. part. adj. *adhaerens* hanging on, sticking to.

Main body of the mother cell is prolate spheroid, ~1–2 μm in diameter, and has one prostheca, 0.2 × 1–5 μm. Buds are motile by a single flagellum. The main body of the reproductive cell, but not the prostheca, is surrounded by capsular polysaccharide. Gram negative. Not acid fast. No endospores. Aerobic. Additional phenotypic characteristics are reported in Table BXII.α.58.

Colonies are round, undulate, about 1.5 mm in diameter after 3 days at 30°C on marine agar. In liquid media, there is granular turbidity due to adhering cell masses, and thick biofilm forms on the surface of the growth vessel. Does not form rosettes. The optimal temperature range for growth is 25–37°C. The ocean salts growth range is 1.5–12%. The optimal pH growth range is 5.7–8.7. Nitrate is reduced. Sheep erythrocytes are not hemolyzed. Susceptible to novobiocin, streptomycin, tellurite, crystal violet, brilliant green, and methylene blue, and resistant to 1.0% Tween 80, penicillin, and ampicillin.

The major fatty acid is octadecanoate (C_{18:1}) at 80% of the total fatty acids (Table BXII.α.59). Isolated in 1982 by J. Smit from an inshore slough in Puget Sound, Pacific Ocean. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 60 (Bd).

Type strain: MHS-3, ATCC 43965.

GenBank accession number (16S rRNA): AF082790.

3. **Hyphomonas hirschiana** Weiner, Devine, Powell, Dagasan and Moore 1985, 242^{VP}

hir'schi.an.a. L. fem. adj. *hirschiana* pertaining to Hirsch; named for P. Hirsch for his contributions to the study of prosthecate, budding bacteria.

Cellular morphology is like that of *H. polymorpha*. Colonies are small (1.0 mm in diameter after 48 h), dull gray, dry, and circular to irregular, with dimpled elevations and lobate margins. Growth in broth produces a pellicle at the surface and a light film of growth on the sides and bottom of the culture vessel.

A mixture of L-glutamic acid, L-aspartic acid, L-serine, and L-methionine is required for growth. The temperature range for optimal growth is 25–31°C. The NaCl concentration for growth is 2.0–15.0%. Optimal pH for growth: 7.6.

Nitrate is reduced. Sheep erythrocytes are not hemolyzed. Susceptible to 0.01% NP-40, novobiocin, and ampicillin. Resistant to 1.0% Tween 80. Intermediate susceptibility to penicillin and streptomycin. Treatment for 9 min or more with a Brownwill Biosonik IV sonicator (low probe at full power) is required to produce 99% lysis of cells.

A major fatty acid is octadecanoate (C_{18:1}) at up to 67% of the total fatty acids (Table BXII.α.59). The type strain was isolated in 1979 from shellfish beds near hydrothermal vents on the floor of the mid-Pacific Ocean by H. Jannasch. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 57 (Bd).

Type strain: VP5, ATCC 33886.

GenBank accession number (16S rRNA): AF082794.

4. **Hyphomonas jannaschiana** Weiner, Devine, Powell, Dagasan and Moore 1985, 240^{VP}

jan' nasch.i.an.a. L. fem. adj. *jannaschiana* pertaining to Jannasch; named for H. Jannasch for his contributions to marine microbiology.

Mature mother cells are 0.5–0.8 × 1.0–3.0 μm, and the main body of the cell tends to be elongated and bullet- or spindle-shaped. Young swarmer cells are motile and pear-shaped and resemble those of other members of the genus. On Zobell marine agar supplemented with 5% sheep blood, colonies are small, dull gray, dry, and circular to irregular with dimpled elevations and lobate margins (strains VP1 and VP3) or gray, circular, convex, entire, glistening, and mucoid (strains VP2^T and VP4), resembling *H. neptunium* colonies, which have similar shape and are off-white. In broth, growth appears granular, and a film of tan cells covers the sides and bottom of the culture vessel (strains VP1 and VP3), or growth may appear ropy and produce only a slight film (strains VP2^T and VP4). Strains VP1 and VP3 tend to form rosette-like cell aggregates.

A brown pigment, identified as a pyomelanin, is produced in type 2216 marine broth at 31–37°C. A mixture containing glutamic acid, aspartic acid, and serine as substrates and the cofactor biotin is required for chemoor-ganotrophic growth. The temperature for optimal growth is 37°C. The NaCl concentration for optimal growth is 3.5–7.5%. Optimal pH for growth: 7.6.

Nitrate is reduced. Produces α-hemolysis on Zobell marine medium supplemented with 5% sheep blood. Susceptible to NP-40 (0.01%), novobiocin, penicillin, ampicillin, and streptomycin. Resistant to Tween 80 (1.0%). Very resistant to breakage by sonication.

A major fatty acid is octadecanoate (C_{18:1}) at up to 65% of the total fatty acids (Table BXII.α.59). Four strains, ATCC 33882 (VP1), ATCC 33883^T (VP2^T), ATCC 33884 (VP3), and ATCC 33885 (VP4), of *H. jannaschiana* were isolated in 1979 from shellfish beds near hydrothermal vents on the floor of the mid-Pacific Ocean. The appearance is typical of Gram-negative bacteria. The only notable fine-structural difference among the strains is the consistent presence of large dark granules in strains VP1 and VP3. The occurrence of these granules is independent of the medium used for culturing. Strains cannot be differentiated on the basis of morphological, serological, nutritional or biochemical tests. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 60 (Bd).

Type strain: VP2, ATCC 33883.

GenBank accession number (16S rRNA): AF082789.

5. **Hyphomonas johnsonii** Weiner, Melick, O'Neill and Quintero 2000, 467^{VP}

john.so' ni.i. M.L. gen. n. *johnsonii* of Johnson, named after the (American) molecular taxonomist John Johnson.

Main body of the mother cell is prolate spheroid, ~1 μm in diameter and has one prosthema, 0.2 × 1 μm. Buds are motile by a single flagellum. Gram negative. Not acid fast. No endospores. Aerobic. Additional phenotypic characteristics are reported in Table BXII.α.58.

Colonies are round, convex, about 1.5 mm in diameter after 3 days at 30°C on marine agar. In liquid medium, growth is granular and/or ropy. May form thin film on growth vessel. Can utilize sugars, in the presence of amino acids, for carbon and energy. The optimal temperature range for growth is 25–37°C. The ocean salts growth range is 1.5–6.0. The pH growth range is 5.7–8.0. Nitrate is reduced. α-Hemolysis in the presence of sheep erythrocytes. Susceptible to novobiocin, penicillin, ampicillin, streptomycin, tellurite, crystal violet, brilliant green; resistant to 1.0% Tween 80, methylene blue, and neutral red.

The major fatty acid is octadecanoate (C_{18:1}) at 64% total fatty acids (Table BXII.α.59). The outer membrane protein profiles, DNA homology, and serology have low but significant similarity to other strains of *Hyphomonas*. Isolated in 1982 by J. Smit from an inshore slough in Puget Sound, Pacific Ocean. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 64 (Bd).

Type strain: MHS-2, ATCC 43964.

GenBank accession number (16S rRNA): AF082791.

6. **Hyphomonas neptunium** (Leifson 1964) Moore, Weiner and Gebers 1984, 71^{VP} (*Hyphomicrobium neptunium* Leifson 1964, 249.)

nep.tu' ni.um. L. n. *neptunus* god of the sea, probably should be L. gen. n. *neptuni*.

See Table BXII.α.58 for characteristics. A major fatty acid is octadecanoate (C_{18:1}) at up to 37% of the total fatty acids (Table BXII.α.59). Isolated from a sample of stored seawater or obtained from the harbor at Barcelona, Spain. The

overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 61.7 (Bd).

Type strain: LE 670, IFAM LE 670, ATCC 15444.

GenBank accession number (16S rRNA): AF082798.

7. ***Hyphomonas oceanitis*** Weiner, Devine, Powell, Dagasan and Moore 1985, 240^{VP}

o.cean.i'tis. N.L. fem. n. *oceanitis* daughter of the ocean.

Cells are round to oval and ~0.9 µm in diameter and usually have one hypha, which is up to 3 times the length of the mother cell. Under favorable growth conditions, a small proportion of cells produce two hyphae, one at each of the poles. Both hyphae are capable of producing buds concurrently. Buds (daughter cells) are pear-shaped and become larger and rounder when they are mature (mother cells) and producing buds. Intercalary buds (i.e., two or more daughter cells remaining attached to the distal pole(s) of the stalk(s) of the mother cell) are common. Buds are motile until hyphae form. Gram negative. Not acid-fast. No endospores. Aerobic or microaerophilic.

On agar, the colonies are colorless, raised, semitranslucent or opaque and, after 3 days, up to 1.5 mm in diameter. In liquid media, growth produces uniform turbidity without a pellicle or sediment. The best growth occurs in media containing sea salts. Does not grow on the mineral salts medium used for *Hyphomicrobium vulgare*. A mixture of L-glutamic acid, L-aspartic acid, L-serine, and L-methionine is required for growth. Requires biotin.

The temperature range for optimal growth is 20–30°C. The NaCl concentration for optimal growth is 1.0–7.5%. Optimal pH for growth is: 7.6. Nitrate is reduced. Sheep erythrocytes are not hemolyzed. Under optimal conditions in marine broth, growth is slower than the growth of other species of *Hyphomonas*. Susceptible to NP-40 (0.01%) and Tween 80 (0.1–1.0%). Resistant to novobiocin, penicillin, ampicillin, and streptomycin. Requires 6–8 min of treatment with a Brownwill Biosonik IV sonicator (low probe at full power) to produce 99% lysis of cells.

A major fatty acid is octadecanoate (C_{18:1}) at up to 60%

of the total fatty acids (Table BXII.α.59). Isolated from the Baltic Sea in 1979 by H. Schlesner. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 59 (Bd).

Type strain: SCH 1325, IFAM 1325, ATCC 33879.

GenBank accession number (16S rRNA): AF082797.

8. ***Hyphomonas rosenbergii*** Weiner, Melick, O'Neill and Qintero 2000, 467^{VP}

ro.sen.ber'gi.i. M.L. gen. n. *rosenbergii* of Rosenberg, named after the (Israeli) microbial ecologist Eugene Rosenberg.

Main body of the mother cell is prolate spheroid, ~1 µm in diameter and has one prostheca, 0.2 × 1 µm. Buds are motile by a single flagellum. Synthesizes a capsule, which surrounds the entire cell at all growth stages, and a polar holdfast, which is temporally synthesized. Gram negative. Not acid fast. No endospores. Aerobic. Additional phenotypic characteristics are reported in Table BXII.α.58.

Colonies are round, convex, about 1.5 mm in diameter after 3 days at 37°C on marine agar. In liquid medium, growth is granular and/or ropy. Biofilm forms on walls of growth vessel. Cells form rosettes. The optimal temperature range is 25–45°C. The ocean salts growth range is 1.0–12.0. The pH growth range is 5.7–8.9. Nitrate is reduced. Sheep erythrocytes are not hemolyzed.

Susceptible to novobiocin, penicillin, ampicillin, streptomycin, tellurite, crystal violet, brilliant green, and methylene blue; resistant to 1.0% Tween 80 and neutral red. The major fatty acid is octadecanoate (C_{18:1}) at 43% total fatty acids. The outer membrane protein profiles, DNA homology, and serology have low but significant similarity to other strains of *Hyphomonas*. Isolated in 1985 from the Guaymas Basin thermal vent region (2000 m deep), Gulf of Mexico by H. Jannasch. The overall DNA–DNA similarities with other *Hyphomonas* species are reported in Table BXII.α.60.

The mol% G + C of the DNA is: 61 (Bd).

Type strain: VP-6, ATCC 43869.

GenBank accession number (16S rRNA): AF082795.

Genus VIII. *Ketogulonicigenium* corrig. Urbance, Bratina, Stoddard and Schmidt 2001, 1068^{VP} (*Ketogulonicigenium* (sic) Urbance, Bratina, Stoddard and Schmidt 2001, 1068)

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Ke.to.gu.lo.ni.ci.ge'ni.um. N.L. n. *acidum ketogulonicum* ketogulonic acid; N.L. neut. suff. *genium* genium that which produces; L. neut. n. *Ketogulonicigenium* that which produces ketogulonic acid.

Gram-negative chemoorganotrophic facultative anaerobes. Cells ovoid or rod-shaped (0.5–0.7 × 0.8–1.3 µm). Oxidase and catalase positive. **Convert L-sorbose to 2-keto-L-gulonic acid.** **Major fatty acids** C_{16:0}, and C_{18:1 ω7c/ω9t/ω12c}. Temperature optimal 27–31°C; pH optimal 7.2–8.5. Grow best on arabinose, glycerol, inositol, lactose, mannitol, and sorbitol.

The mol% G + C of the DNA is: 52.1–54.

Type species: ***Ketogulonicigenium vulgare*** Urbance, Bratina, Stoddard and Schmidt 2001, 1069 (*Ketogulonicigenium vulgare* (sic) Urbance, Bratina, Stoddard and Schmidt 2001, 1069.)

FURTHER DESCRIPTIVE INFORMATION

Analysis of 16S rDNA sequences placed the two species of the genus *Ketogulonicigenium* in the family *Rhodobacteraceae* of the *Alphaproteobacteria*, distinct from a cluster containing the genera *Gluconobacter* and *Acetobacter*. The five strains of *Ketogulonicigenium* were phenotypically similar but could be separated into two species on the basis of DNA/DNA hybridization; the five strains include four new isolates and DSM 4025, which was previously classified as *Gluconobacter oxydans* (Urbance et al., 2001).

ENRICHMENT AND ISOLATION PROCEDURES

Enrichments were carried out in liquid medium with L-sorbose as the predominant carbon source and with CaCO_3 (6 g/l) added to buffer the medium against acidic metabolic products (Urbance et al., 2001). Culture supernatants were examined for 2-keto-L-gulononic acid production by TLC and HPLC; positive cultures were serially diluted and spread on agar of the same composition as the enrichment medium. Individual colonies were

purified by streaking and then tested for the ability to produce 2-keto-L-gulononic acid by TLC and HPLC (Urbance et al., 2001).

MAINTENANCE PROCEDURES

Isolates were maintained on trypticase soy agar or another complex medium, SYM agar, described in Urbance et al. (2001). Long-term storage was achieved by freezing at -70°C in peptone-yeast extract-mannitol medium amended with 20% glycerol (Urbance et al., 2001).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *KETOGULONICIGENIUM*

Ketogulonigenium vulgare is nonmotile, does not grow at 37°C , has a pH optimum of 7.2–8.0, and grows best at a sodium ion concentration of 31.2 mM. *Ketogulonigenium robustum* is motile,

grows at 37°C , has a pH optimum of 8.0–8.5, and grows best at sodium ion concentrations between 117–459 mM (Urbance et al., 2001).

List of species of the genus *Ketogulonigenium*

1. ***Ketogulonigenium vulgare*** Urbance, Bratina, Stoddard and Schmidt 2001, 1069^{VP} (*Ketogulonigenium vulgare* (sic) Urbance, Bratina, Stoddard and Schmidt 2001, 1069.)
vul.ga' re. L. neut. adj. *vulgare* common.

Description as for the genus with the following additional characteristics. Nonmotile. No growth at 37°C . pH optimum 7.2–8.0. Optimum Na^+ concentration for growth 31.2 mM.

The mol% G + C of the DNA is: 53.4–54.0 (T_m).

Type strain: : DSM 4025 (patent strain).

GenBank accession number (16S rRNA): AF136849.

2. ***Ketogulonigenium robustum*** Urbance, Bratina, Stoddard and Schmidt 2001, 1069^{VP} (*Ketogulonigenium robustum* (sic) Urbance, Bratina, Stoddard and Schmidt 2001, 1069.)
ro.bus' tum. L. adj. *robustum* strong.

Description as for the genus with the following additional characteristics. Motile. Grow at 37°C . pH optimum 8.0–8.5. Optimum Na^+ concentration for growth 117–459 mM.

The mol% G + C of the DNA is: 52.1 (T_m).

Type strain: X6L, KCTC 0858BP, NRRL B-21267.

GenBank accession number (16S rRNA): AF136850.

Genus IX. *Maricaulis* Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennisar, Smit and Tesar 1999, 1071^{VP}

JEANNE S. POINDEXTER

Ma' ri. cau' lis. L. neut. n. *mare* the sea; L. masc. n. *caulis* stalk; M.L. masc. n. *Maricaulis* stalk from the sea.

Cells single, unbranched, with poles gently rounded or slightly tapered; long axis typically straight and cell shape **bacteroid**, but may be slightly curved in some cells and some isolates and cell shape **subvibrioid**; $0.4\text{--}0.6 \times 1\text{--}2 \mu\text{m}$ during growth in most media. The younger pole of the dividing cell bears a **single flagellum**, and the older pole bears a prostheca (the **stalk**) developed by outgrowth of the cell envelope. Stalk diameter is constant along its length, varying from 0.11 to 0.18 μm among isolates. Binary **fission is asymmetric**: prior to fission, one pole bears a stalk and the other a single flagellum, and fission results in the separation of a non-motile stalked cell and a motile, monoflagellate swarmer cell. At the base of the flagellum and at the outer tip of the stalk is a small mass of adhesive material, the **holdfast**, which confers adhesiveness on each of the progeny.

Gram negative. All isolates grow aerobically with O_2 as terminal electron acceptor; principal respiratory quinone is ubiquinone-10. Nitrate may be reduced to nitrite anaerobically; N_2 is not produced. Some isolates (12 of 25 tested) can grow anaerobically on peptone-yeast extract medium without additional carbohydrates. Colonies are circular, convex and glistening, with a smooth margin, butyrous in texture, and colorless. In unagitated liquid cultures, cells accumulate as a **surface film** or pellicle and develop as a ring of growth on the vessel wall at or just below the air-liquid interface. Growth in agitated liquid cultures is evenly dispersed.

Chemoorganotrophic and **oligotrophic**; grow optimally in media such as peptone-yeast extract containing 0.05–0.3% (w/v) organic solutes prepared in sea water or artificial sea salts solution, but not in undiluted standard marine broth such as Zobell's medium 2216, in which growth is inhibited or cells are deformed and viability is low. Optimal temperature range for growth 20–25°C; tolerated range for growth 10–35°C. Optimal pH for growth near neutrality; pH 6–8 tolerated. Growth in peptone or Casamino acids media requires the addition of sea salts or NaCl; optimal NaCl concentration for growth 2–6% (w/v); tolerated range 0.5–8% (w/v). Maximum specific rates of exponential growth $0.17\text{--}0.23 \text{ h}^{-1}$. Do not grow in defined media, and growth is not stimulated by B vitamins; almost all isolates require unidentified growth factors available in peptone. Do not use alcohols as sole sources of carbon.

Comparative analysis of 16S rDNA sequences is consistent with placement of *Maricaulis* among the *Alphaproteobacteria*. The positions of the 15 sequences grouped as *Maricaulis* in Fig. BXII.α.111 of the chapter describing the genus *Caulobacter* (see the family *Caulobacteraceae*) are represented here in Fig. BXII.α.76 by the seven nonredundant sequences with their strain identities and GenBank accession numbers shown. These seven sequences were determined for isolates known to exhibit *Maricaulis* phenotypes. The positions of the sequences for *Hirschia baltica*, “marine caulobacter” isolate MCS23, and the six nonredundant se-

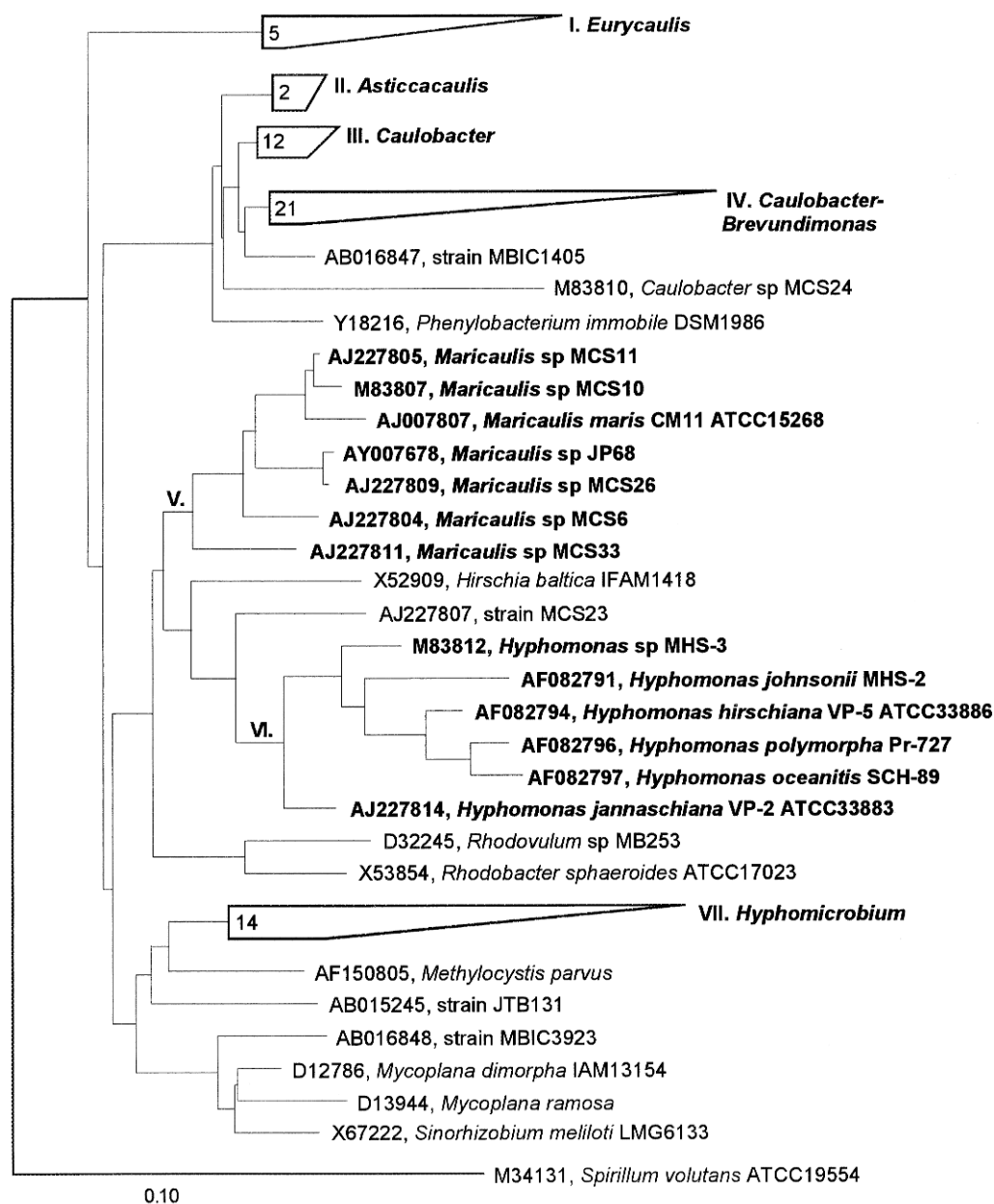


FIGURE BXII.α.76. Phylogenetic tree of 81 16S rDNA sequences, with group V. *Maricaulis* and group VI. *Hyphomonas* expanded to display GenBank accession numbers and strain identities (see Fig. BXII.α.114 in the chapter on the genus *Caulobacter*). Also represented are the positions of *Maricaulis* sequences AJ227810 (by AJ227805); AJ007804, AJ227802, and AJ227803 (by AJ007807); AJ227808 (by AJ227809); and AJ227806, M83809, and M83811 (by AJ227811). Represented *Hyphomonas* sequences are AF082795 and AF082798 (by AF082794); AJ227813 (by AF082796); AF082789, AF082790, AF082792, AF082793, and AF082799 (by AJ227814); and M83806 (by M83812). Bar indicates evolutionary distance.

quences among the 15 reported for *Hyphomonas* spp. are also displayed to illustrate their similarity to *Maricaulis* sequences. (See "Taxonomic Comments" in the chapter in which the genus *Caulobacter* is described.)

Sources: isolated from filtered, stored seawater and from natural bodies of seawater. Not encountered as clinical isolates, and not known to be pathogenic for plants or animals.

The mol% G + C of the DNA is: 62.5–64.0.

Type species: *Maricaulis maris* (Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancannet, Tindall,

Bennasar, Smit and Tesar 1999, 1071 (*Caulobacter maris* Poindexter 1964, 289.)

FURTHER DESCRIPTIVE INFORMATION

Morphology and fine structure Known isolates of *Maricaulis* are either bacteroid or subvibrioid, i.e., cell poles are gently rounded or tapered, and the long axis is not strongly curved. However, vibrioid caulobacters are seen in samples and enrichment cultures of seawater, and the present description should not prevent an isolate from being identified provisionally as *Mari-*

caulis. Vibrioid marine isolates have been obtained from deep-sea vent communities (Poindexter, unpublished), but further characterizations are required to determine whether they are otherwise significantly different from isolates on which the present description is based.

As in freshwater caulobacters, a holdfast is present at the distal tip of the *Maricaulis* stalk; the holdfasts of all adhesive marine strains tested bound Wheat Germ Agglutinin (Merker and Smit, 1988; Abraham et al., 1999; 2001) and so must contain some *N*-acetylglucosamine. This is a higher proportion of WGA-binding strains than was found for freshwater caulobacters, suggesting that the composition of holdfast material may be constrained by the ionic environment of the marine habitat.

Maricaulis isolates have not been examined in ultrathin sections to determine the ultrastructure of the stalk. Although these marine bacteria are susceptible to disintegration during preparation for electron microscopy of whole cells, the slenderness of the stalks on the cells that do remain intact during specimen preparation and the absence of budding from the tip of the prostheca are features consistent with an internal structure comparable to that of freshwater caulobacters (see Morphology in the chapter on the genus *Caulobacter*). In contrast to freshwater caulobacters, however, the stalks of *Maricaulis* isolates do not contain stalk bands. An S-layer coat has not been discerned in *Maricaulis* cells, but their cell surface is poorly preserved by the procedures that have been used to study this feature in *Caulobacter* cells.

Cellular composition The principal phospholipid in *Maricaulis* cells is phosphatidylglycerol, and the most abundant fatty acid species are $C_{18:1}$ and $C_{16:0}$. All isolates tested also contain lower, but significant, amounts of $C_{18:1\ \omega 7c\ 11CH_3}$ and species of $C_{17:1}$ and $C_{16:1}$, $C_{17:0}$, and—in contrast to freshwater caulobacters— $C_{18:0}$ and $C_{11:0\ iso\ 3OH}$ (Abraham et al., 1999; Abraham et al., 2001). Phospholipids are significantly less abundant than in freshwater caulobacters and most other bacteria, and phosphoglycerolipids are not detectable. All seven isolates so far analyzed possess sulfoquinovosyl diacylglycerols, one of which was identified as 1-O-(α -6',6-deoxy-aldoheptopyranosyl-6'-sulfonic acid)-3-O-diacylglycerol, and four also formed at least small amounts of taurineamide diacylglycerols (Abraham et al., 1999). This pattern of cellular lipids is similar to that found by the same laboratory for *Hyphomonas* isolates, but differs noticeably by the absence of sulfoquinovosyl diacylglycerides from the cells of these budding, hyphal bacteria and of one isolate (MCS23) regarded as a caulobacter throughout its isolation. This is lipid pattern "M" of Abraham et al. (1997).

Genome structure Native plasmids were detected in two marine isolates by Anast and Smit (1988), but not in the majority of strains. More significantly for potential employment of these organisms in marine habitats, every isolate tested was able to propagate and express genes (for antibiotic resistance) carried on plasmids transferred to the marine caulobacters by conjugation with *Escherichia coli* donors. Ribosomal DNA sequences have been determined for a large number of *Maricaulis* and *Maricaulis*-like isolates. *Maricaulis* sequences constitute one of five coherent groups among caulobacterial sequences. The minimum similarity value among sequences grouped within V. *Maricaulis* in Fig. BXII.α.76 is 92.2%. The highest similarities outside the genus are to sequences for organisms identified as *Hyphomonas jannaschiana* (90.6–92.0%). Two sequences are similar to *Maricaulis* and *H. jannaschiana* sequences: the sequence for *Hirschia baltica* is up to 89.8% similar to *Maricaulis* sequences, and the

sequence for isolate MCS23, described as a caulobacter, is up to 89.5% similar. Bacteriophages lytic for *Maricaulis* isolates have not been reported. (See "Taxonomic Comments" below and the chapter on the genus *Caulobacter* for further discussion of 16S rDNA sequences and other bacteriophages.)

Antibiotic and metal susceptibilities *Maricaulis* isolates are typically susceptible to most antibiotics tested. However, nine of 20 isolates tested were found resistant to 10 µg/ml or more of $HgCl_2$. Mercury resistance was not correlated with the presence of plasmids, and a probe could not detect the mercury reductase gene.

ENRICHMENT AND ISOLATION PROCEDURES

Maricaulis isolates are obtained by the basic procedure described for *Caulobacter* (see the chapter on the genus *Caulobacter*), beginning with a sample of estuarine or seawater. Deep-sea *Maricaulis* isolates were obtained by allowing glass cover slips to reside in an open box for several weeks near a deep-sea vent; caulobacters attached to the glass surfaces, which were used as inocula for enrichment cultures in dilute peptone-seawater medium (Poindexter, unpublished). Once isolated, *Maricaulis* strains can be maintained on slants of dilute peptone (0.05%, w/v)–Casamino acids (0.05%, w/v) media prepared with 75–80% seawater or artificial sea salts solution, transferred every two months, incubated for 2–3 d at room temperature, then refrigerated. Lyophilization is not a suitable means of preservation.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

The distinctive features of these bacteria are morphological—the asymmetry of the dividing cell, the single polar flagellum, the holdfast, and the absence of bands in the stalks. However, intact cells are difficult to preserve for the electron microscopical examinations needed for close examination of these features. Aldehyde preservation is effective with some isolates, and acetic acid (2%, v/v) for others, but many disintegrate during specimen preparation. Close observation by phase-contrast microscopy of wet cells is, however, adequate to determine the asymmetry of dividing cells, the presence of stalks, and adhesiveness and motility of cells.

DIFFERENTIATION OF THE GENUS *MARICAULIS* FROM OTHER GENERA

Four groups of bacteria other than caulobacters produce long cellular appendages of the envelope that are of constant diameter; these bacteria also tend to occur in habitats with caulobacters. They are distinguishable from caulobacters, and from each other, by the morphology of their reproductive stages. The five groups are distinguished as in Table BXII.α.101, and the five species groups of caulobacters are distinguished as shown in Table BXII.α.102, both in the chapter in which the *Caulobacter* is described. Relationships to nonprosthecate bacteria implied by 16S rDNA sequence analysis are illustrated and discussed further in "Taxonomic Comments" in the chapter describing the genus *Caulobacter*.

TAXONOMIC COMMENTS

Among the 16S rDNA sequences that were found similar to those of *Maricaulis* isolates, the entire set determined for *Hyphomonas* isolates is clearly the most similar (more than 90% similarity), while the sequences of freshwater caulobacters are less similar to *Maricaulis* sequences than are those of phototrophic bacteria such as *Rhodobacter* and *Rhodopseudomonas*. This phylogenetic re-

lationship could reflect a common marine ancestry of *Maricaulis* and *Hyphomonas* in the ocean, an ancestry further implied by the similarity of lipid composition of these two groups. In each morphological type, the marine isolates are more like their co-habitants than like their freshwater/soil morphological twins. It is not so easy to distinguish these two kinds of dimorphic, prosthecae bacteria upon first observing them, even in pure cultures. For example, the "VP" designation on several *Hyphomonas* strains was used to label deep-sea Vent Prosthecae bacteria until morphology could be established unequivocally for pure populations (Poindexter, unpublished). Among the marine caulobacters collected by Anast and Smit (1988), one isolate (MCS23) appears closer, by several criteria, to *Hyphomonas* than to any isolates within the coherent *Maricaulis* group. This isolate might reflect

TABLE BXII.α.61. Differential characteristics of the species of the genus *Maricaulis*^a

Characteristic	<i>M. maris</i>	" <i>M. halobacteroides</i> "
Growth with 4% NaCl	+	—
Carbon sources generally used:		
Carbohydrates	+	+
Amino acids	—	+
Other organic acids	—	+
Starch hydrolyzed	—	+
Nitrate reduced to nitrite anaerobically	+	—

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative.

a shift in reproductive function between prostheca and cell, but the direction in which it might be evolving cannot be inferred.

Two species are recognized, distinguished by physiological traits as shown in Tables BXII.α.61 and BXII.α.62..

TABLE BXII.α.62. Other characteristics of the species of the genus *Maricaulis*^a

Characteristic	<i>M. maris</i>	" <i>M. halobacteroides</i> "
Carbon-source utilization: ^b		
Arabinose	—	+
Ribose	—	+
Xylose	+	+
Glucose	+	+
Galactose	—	+
Mannose	—	+
Lactose	—	+
Maltose	+	+
Sucrose	+	+
Proline	—	+
Acetate	—	+
Butyrate	—	+
Pyruvate	—	+
Starch hydrolysis	—	+

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative. Neither species utilizes any of fructose, alanine, aspartate, glutamate, tyrosine, pimelate, malate, fumarate, or succinate when provided as sole source of carbon in dilute (0.005%, w/v, of each of peptone and yeast extract) media prepared with mineral nutrients and 3% NaCl.

^bCarbon-source utilizations were determined with D-isomers of sugars, with L-isomers of amino acids, or (when single isomers were not available) with racemic mixtures.

List of species of the genus *Maricaulis*

1. ***Maricaulis maris*** (Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Ben-nasar, Smit and Tesar 1999, 1071^{VP} (*Caulobacter maris* Poindexter 1964, 289.)
ma'ris. L. gen. n. *maris* of the sea.

Rod-shaped cells slender. Colonies colorless. Organic growth factor requirements not satisfied by mixtures of B vitamins and amino acids. Growth requires NaCl (1–4%, w/v). Known isolates utilize few sugars and do not hydrolyze starch; they do not utilize individual amino acids as carbon sources, but grow on peptone-Casamino acids media.

Strain CM11 was isolated from filtered, stored seawater in Pacific Grove, CA.

The mol% G + C of the DNA is: 62.5 (HPLC).

Type strain: CM11, ACM 5106, ATCC 15268, DSM 4734.

GenBank accession number (16S rRNA): AJ227802.

Additional Remarks: One of two marine species included in this list. Vibrioid marine and estuarine isolates, also dependent on NaCl supplementation for growth in complex organic media, are known; their characteristics have not been reported. At least some of the isolates of marine caulobacters reported by Anast and Smit (1988) could be assigned here, but others may require accommodation in another species. Some of those isolates appear more subvibrioid than bacteroid, but whether cell morphology will serve as a suitable subgeneric criterion among *Maricaulis* isolates has yet to be determined.

Other Organisms

1. "*Maricaulis halobacteroides*" (*Caulobacter maris* Poindexter 1964, 289.)

hal.o.bac.ter.oi'des. Gr. n. *hals* salt; M.L. masc. *bacter* Gr. neut. n. *bactrum* rod; Gr. n. *eidus* form, shape; M.L. adj. *halobacteroides* salt (-needing) and rod shaped.

Rod-shaped cells slender. Colonies colorless. Organic growth factor requirements not satisfied by mixtures of B vitamins and amino acids. Starch is hydrolyzed, and most

sugars, but few individual amino acids, can be utilized as sole sources of carbon for growth. Growth requires NaCl (0.5–3%, w/v) and is inhibited at 4% (w/v) NaCl.

Strain CM13 was isolated from filtered, stored seawater in Pacific Grove, CA.

The mol% G + C of the DNA is: not determined.

Deposited strain: CM13, ATCC 15269.

GenBank accession number (16S rRNA): AJ007804.

Additional Remarks: The original type strain deposited with the ATCC appears to have been, very early, overgrown by strain CM11 (*M. maris*), resulting in identity of the 16S rDNA sequences that have been determined for the two strains (see Fig. BXII.α.76) and DNA–DNA hybridization >90% (Moore et al., 1978; Abraham et al., 1997). Other

copies of the ATCC strain (DSM 4734 and ACM 5106) may also be strain CM11. The problem probably arose from attempts to maintain the strains as lyophilized suspensions, a state from which it is difficult to revive these marine bacteria. At present, it seems wiser to continue to recognize the species and thereby possibly provide a taxon suitable for recent isolates of this type of marine caulobacter.

Genus *X. Methylocarcula* Doronina, Trotsenko, and Tourova 2000a, 1857^{VP}

NINA V. DORONINA AND YURI A. TROTSENKO

Me.thyl.ar.cu'la. Fr. *méthyle* the methyl radical; L. fem. n. *arcula* small box; M.L. fem. n. *Methylocarcula* methyl-using small box.

Rods 0.5–0.8 × 0.8–2.0 μm. Nonmotile. Gram negative. **Poly-β-hydroxybutyrate granules are formed.** Endospores and prosthecae are not formed. Colonies are white or pale pink. Do not produce pyocyanin and fluorescein. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Moderately halophilic**; NaCl is required for growth. Ectoine is accumulated intracellularly as the main osmoprotectant. Growth does not occur on peptone/yeast extract medium, with or without NaCl. Nitrate is not reduced to nitrite. Chemoorganotrophic. **Facultatively methylotrophic. Assimilate C₁ compounds via the isocitrate lyase-negative (icl[−]) serine pathway. Methylamine, sugars, and some organic acids are used as carbon and energy sources.** Ammonium salts, some amino acids, and methylamine are used as nitrogen sources. Growth factors are not required. **Oxidase positive.** Acids are produced from sugars oxidatively. Acetoin, hydrogen sulfide, and ammonium are not formed. Indole is formed from L-tryptophan. The major ubiquinone is Q-10. The dominant phospholipids are phosphatidylethanolamine and phosphatidylcholine. The predominant cellular fatty acids are straight-chain unsaturated (C_{18:1}), saturated (C_{18:0}), and cyclopropane (C_{19:0}) acids. Belongs to the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 57–60.

Type species: *Methylocarcula marina* Doronina, Trotsenko and Tourova 2000a, 1858.

FURTHER DESCRIPTIVE INFORMATION

Cells are lysed when transferred into distilled water after NaCl treatment. The divalent cations Mg²⁺ and Ca²⁺ added to the 0.5 M NaCl washing solution at concentrations as low as 50 mM prevent cell lysis. The ectoine pool increases when the NaCl concentration in the growth medium is increased.

Methylamine dehydrogenase and amine oxidase are absent in methylamine-grown cells. Alternatively, they contain an inducible γ-glutamylmethylamide synthetase/lyase and N-methylglutamate synthase/lyase. Both enzyme systems produce formaldehyde, which is further oxidized by glutathione-independent formaldehyde dehydrogenase to formate. The latter is ultimately oxidized to CO₂ by phenazine methosulfate-linked and NAD-dependent formate dehydrogenases. Formaldehyde assimilation occurs via the serine pathway as confirmed by the presence of the appropriate specific enzymes: hydroxypyruvate reductase, serine-glyoxylate aminotransferase, and malate lyase. *Methylocarcula* strains either lack or have very low isocitrate lyase (icl[−]) activity and during growth on C₁ compounds consequently implement

the icl[−] variant of the serine pathway. Primary assimilation of ammonia occurs both by reductive amination of α-ketoglutarate and via the glutamate cycle.

ENRICHMENT AND ISOLATION PROCEDURES

Successful enrichment has been achieved by inoculating water or soil samples with a salinity of 6–11% (pH 6.5–7.5) into mineral medium MK¹. Enrichment cultures were grown for 1 week at 29°C in 750-ml Erlenmeyer flasks containing 100 ml of the medium, with shaking at 120 rpm; they were then diluted 1:10³ and plated on solidified MK medium containing 2.0% Bacto agar (Difco).

MAINTENANCE PROCEDURES

Methylocarcula cultures can be stored for 2 months on MK agar slants at 4°C. For long-term preservation, *Methylocarcula* strains can be lyophilized in skim milk by using common procedures for aerobes.

DIFFERENTIATION OF THE GENUS *METHYLARCULA* FROM OTHER GENERA

The major characteristics differentiating the genus *Methylocarcula* from other related genera are summarized in Table BXII.α.63.

TAXONOMIC COMMENTS

In the phylogenetic tree derived from 16S rRNA sequences, *Methylocarcula* is located in the order *Rhodobacterales* in the class *Alphaproteobacteria*, whereas most other genera of methyllobacteria having the serine pathway belong to the order *Rhizobiales* in the class *Alphaproteobacteria*. The moderately halophilic marine genus *Methylophaga*, which uses the ribulose monophosphate pathway, belongs to the class *Gammaproteobacteria*. The similarity values for species *Methylocarcula* and other members of the order *Rhodobacterales* in the class *Alphaproteobacteria*, including the marine methylotroph “*Marinosulfonomonas*” (Holmes et al., 1997), fell into the range 87.1–92.6%.

Methylocarcula has less than 10% DNA–DNA similarity with members of *Methylorhabdus*, *Methylobacterium*, *Aminobacter*, and *Methylopila*.

1. MK medium contains (g/l of distilled water): (NH₄)₂SO₄, 2.0; KH₂PO₄, 2.0; NaCl, 60.0; MgSO₄·7H₂O, 0.2 g; FeSO₄·7H₂O, 0.002 (Doronina et al., 2000a). The pH of the medium was adjusted to 7.2 prior to autoclaving. Methylamine was added to a final concentration of 3.0 g/l.

TABLE BXII.α.63. Major differentiating characteristics of facultative serine pathway methyllobacteria belonging to various genera^a

Characteristic	<i>Methylarcula</i>	<i>Aminobacter</i>	<i>Hyphomicrobium</i>	<i>"Marinosulfonomonas"</i>	<i>Methylbacterium</i>	<i>Methylophila</i>	<i>Methylorhabdus</i>	<i>"Methylsulfonomonas"</i>
Morphology (flagella)	—	+	+	—	+	+	—	+
Reproduction by:								
Budding	—	+	+	—	—	—	—	—
Division	+	—	—	+	+	+	+	+
Hyphae formation	—	—	+	—	—	—	—	—
Oxidase	+	+	—	+	+	+	—	—
Catalase	±	+	+	+	+	—	+	+
Carotenoids	—	—	—	—	+	—	—	—
Reduction of NO ₃ to NO ₂	—	+	+	nd	+	+	+	nd
Methylamine metabolism:								
Amine dehydrogenase or	—	—	—	nd	+	+	+	nd
<i>N</i> -methylglutamate derivatives	+	+	+	nd	+	—	—	nd
γ-Glutamylmethylamide lyase	+	—	—	nd	—	—	—	nd
Isocitrate lyase	—	+	+	nd	—	—	—	nd
Cyclopropane acid, C _{19:0} cyclo	+	+	+	+	Trace	+	+	+
Methyloctadecenoic acid, C _{18:1} ω7 CH ₃	—	—	+	nd	—	+	+	nd
Major ubiquinone	Q-10	Q-10	Q-9	nd	Q-10	Q-10	Q-10	nd
Tolerance to NaCl (%)	12	2.5	3	3.5	2.5	2	2	0.5
Growth at pH 10.0	+	—	—	—	—	—	—	—
Utilization of methanol	—	—	+	+	+	+	+	+
Mol% G + C of DNA	57–61	62–64	61–65	57	60–70	66–70	66–67	61

^aSymbols: +, present; —, absent; ±, variable; nd, not determined.*List of species of the genus Methylarcula*1. ***Methylarcula marina*** Doronina, Trotsenko and Tourova 2000a, 1858^{VP}*ma.ri'na*. L. fem. n. *mare* sea; L. fem. adj. *marina* of the sea.

Fig. BXII.α.77 illustrates the morphological features. Colonies on methylamine agar medium are white or pale pink, round, convex, 1–2 mm in diameter. Oxidase positive. Urease, lipase, and catalase negative. Nitrate is not utilized. Able to grow at 10–42°C, at pH 5.0–10.5, and in the presence of 0.05–12% NaCl. Optimal conditions for growth are 29–35°C, pH 7.5–8.5, and 3–8% NaCl. Utilizable carbon sources are methylamine, fructose, glucose, maltose, lactose, mannose, ribose, trehalose, galactose, xylose, sucrose, succinate, pyruvate, and acetate. Methylamine is oxidized to formaldehyde by the *N*-methylglutamate pathway enzymes γ-glutamylmethylamide synthetase/lyase and *N*-methylglutamate synthase/lyase.

The type strain was isolated from Azov Sea estuary water. The mol% G + C of the DNA is: 60.4 (*T_m*).

Type strain: h1, VKM B-2159.

GenBank accession number (16S rRNA): AF030437.

2. ***Methylarcula terricola*** Doronina, Trotsenko and Tourova 2000a, 1858^{VP}*ter.ri'co.la*. Gr. adj. *terricola* of the soil.

Fig. BXII.α.77 illustrates the morphological features. Colonies on methylamine agar medium are white, 2 mm in diameter. Oxidase and catalase positive. Urease and lipase negative. Nitrate is not utilized. Able to grow at 10–40°C, pH 5.5–10.0, and in the presence of 0.05–14% NaCl. Optimal conditions for growth are 29–32°C, pH 7.5–8.5, and 3–6% NaCl. Utilizable carbon sources are mono- and dimethylamine, fructose, glucose, maltose, lactose, mannose, ribose, trehalose, galactose, xylose, sucrose, succinate, pyruvate, and acetate. Methylamine is oxidized to formaldehyde by the *N*-methylglutamate pathway enzymes γ-glutamylmethylamide synthetase/lyase and *N*-methylglutamate synthase/lyase.

The level of DNA–DNA similarity with the type species *Methylarcula marina* is ~25–30% (membrane method).

The type strain was isolated from coastal salty soil of the Black Sea (Crimea).

The mol% G + C of the DNA is: 57.1 (*T_m*).

Type strain: h37, VKM B-2160.

GenBank accession number (16S rRNA): AF030436.

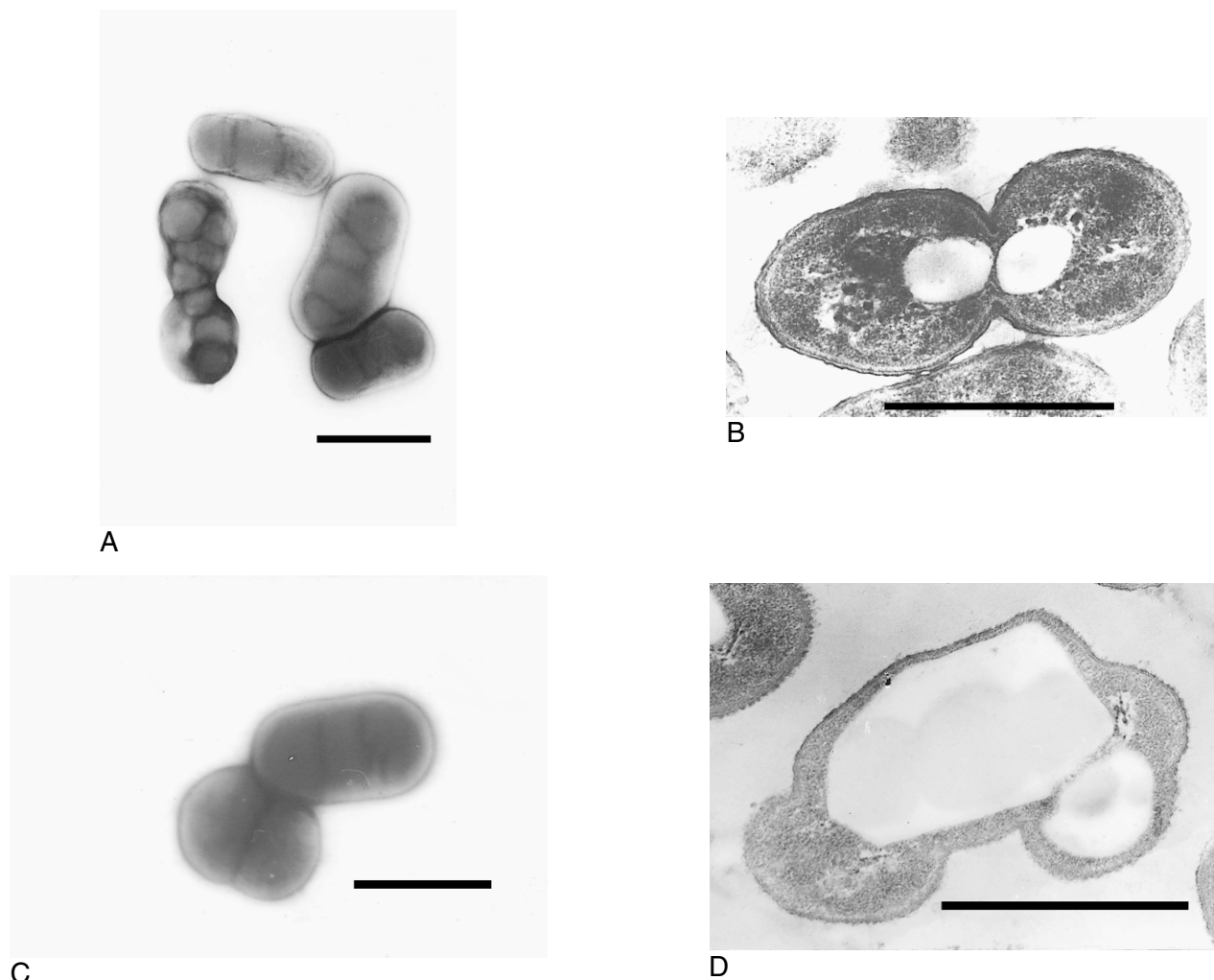


FIGURE BXII.α.77. *Methyloarcula marina* (A, B) and *Methyloarcula terricola* (C, D). A and C, negatively stained cells; B and D, ultrathin sections showing cell wall structure and granules of poly-β-hydroxybutyrate. Bars = 1 μm.

Genus XI. Octadecabacter Gosink, Herwig and Staley 1998, 327^{VP} (Effective publication: Gosink, Herwig and Staley 1997, 363)

JOHN J. GOSINK

Oc.ta.dec'a.bac.ter. Gr. pref. *okto* eight; Gr. pref. *deca* ten; Gr. neut. n. *bakterion* a rod; M.L. masc. n. *Octadecabacter* an 18-carbon fatty acid-containing rod.

Straight or pleomorphic rods 0.6–0.8 × 1.6–4.8 μm. Nonmotile.

All known strains produce gas vesicles. Gram negative. Aerobic or microaerophilic, possessing a strictly respiratory type of metabolism, with oxygen as the terminal electron acceptor. Do not reduce NO₃[−] to NO₂[−]. Do not grow anaerobically. Do not produce bacteriochlorophyll *a*. Form white, circular, convex, entire colonies on SWCm agar.¹ Growth occurs at pH 6.5–8.5 and at

temperatures as low as 4°C in media having 17–70‰ salinity (4.8% NaCl). **Catalase positive. Oxidase negative.** Growth occurs on few (if any) organic compounds as sole carbon sources at 0.2% concentration. Growth occurs sparingly on selected carbon sources, including L-glutamate, glycerol, and mixed amino acids in dilute yeast extract. **Octadecenoic acid (C_{18:1}) is the major fatty acid.** Member of the class *Alphaproteobacteria*. Isolated from polar marine sea ice.

The mol% G + C of the DNA is: 56–57 (HPLC).

Type species: **Octadecabacter arcticus** Gosink, Herwig and

1. SWCm agar (Irgens et al., 1989) has the following composition (g per l of distilled water): NaCl, 12.0; MgSO₄·7H₂O, 7.0; MgCl₂·6H₂O, 5.2; CaCl₂·2H₂O, 1.1; KCl, 0.7; KH₂PO₄, 0.01; ferric citrate, 0.001; NH₄Cl, 0.4; yeast extract, 0.4; beef extract, 0.4; tryptone, 0.4; vitamins (mg per l of distilled water): pyridoxine·HCl, 10; calcium pantothenate, 5; nicotinamide, 5; *p*-aminobenzoic acid, 5; riboflavin, 5; thiamine·HCl, 5; biotin, 2; folic acid, 2; cyanocobalamin (B₁₂), 0.1; 10 ml; trace elements solution ((g per l of distilled water): H₃BO₃, 0.2; CaCl₂·2H₂O, 0.2;

ZnSO₄·7H₂O, 0.1; MnCl₂·4H₂O, 0.03; Na₂MoO₄·2H₂O, 0.03; NiCl₂·6H₂O, 0.02; CuCl₂·2H₂O, 0.01; adjust pH to 3.5.), 2.0 ml; agar, 15; adjust pH to 7.0 before sterilizing.

Staley 1998, 327 (Effective publication: Gosink, Herwig and Staley 1997, 363.)

FURTHER DESCRIPTIVE INFORMATION

Octadecabacter strains appear as rods or pleomorphic rods that form small, white, circular, convex, entire colonies when grown on SWCm agar plates at 4°C for several weeks. These strains do not produce bacteriochlorophyll *a* or any associated pigments.

All known *Octadecabacter* strains produce gas vesicles. The role of gas vesicles in this genus is unknown. Gas vesicles may serve to buoy cells in the water column up to the sea ice microbial community. Alternatively, gas vesicles may serve as a mechanism for cell dispersal during spring breakup of the sea ice.

Octadecabacter (and some related genera) are distinguished by a high level of octadecenoic acid: 70–80% of the total cellular fatty acids (Gosink and Staley, 1995). The location and *cis* or *trans* nature of the double bond in the octadecenoic acid (either C_{18:1} ω₇, C_{18:1} ω₉, or C_{18:1} ω₁₂) of *Octadecabacter* has not been determined. The remainder of the fatty acids are mostly C_{16:1} ω₇, C_{16:0}, and C_{10:0} 3OH and several other fatty acids, each of which constitutes 3% or less of the total.

The strains grow on few (if any) organic compounds as sole carbon sources at 0.2% concentration. However, limited growth occurs when 0.2 g/l yeast extract is added to the basal medium. All of the polar gas vacuolate strains grow on L-glutamate, glycerol, and mixed amino acids in the presence of 0.2 g/l yeast extract.

Phylogenetically closely related taxa have been isolated from a number of marine environments around the world. The physiological capabilities and ecological roles of these bacteria are quite diverse (Sorokin, 1995; Bowman et al., 1997a; Gonzáles and Moran, 1997; Labrenz et al., 1999).

ENRICHMENT AND ISOLATION PROCEDURES

All currently known strains have been obtained from polar marine sea ice. Strains can be collected by melting ice fragments at room temperature several hours to overnight. The meltwater is then plated on suitable media and incubated at 4°C. *Octadecabacter* has only been cultivated from natural environments by plating on SWCm medium; however, media using several other

carbon sources may also work. In general, only dilute carbon sources should be used. *Octadecabacter* colonies are usually only seen as small white colonies after several weeks of growth. The presence of gas vesicles gives the colonies a chalky opaque coloration. The occurrence of gas vesicles can be confirmed by phase and electron microscopy. *Octadecabacter* strains are readily distinguished from other gas vacuolate strains by their color, slow growth, and fatty acid composition.

MAINTENANCE PROCEDURES

Stocks are maintained for general work on SWCm at 4°C. Care must be taken when transferring or working with the strains to keep them and the growth media at 4°C. Cultures can be killed by leaving them out at room temperature for several hours or overnight. Long-term storage is best done by transferring cells to SWCm broth with 25% glycerol or 10% DMSO and freezing at –80°C.

DIFFERENTIATION OF THE GENUS *OCTADECABACTER* FROM OTHER GENERA

In terms of phenotypic characteristics, *Octadecabacter* can be differentiated from *Roseobacter* and *Sulfitobacter* primarily on the basis of fatty acid composition, lack of pigmentation, lack of bacteriochlorophyll *a*, and gas vesicles (Table BXII.α.64).

Octadecenoic acid (C_{18:1}) comprises a reproducibly high proportion (70–80%) of the total cellular fatty acids of *Octadecabacter* (Gosink and Staley, 1995). However, this fatty acid is found in relative abundance in both pigmented and nonpigmented, bacteriochlorophyll *a*-producing and nonbacteriochlorophyll *a*-producing, psychrotrophic, and mesophilic, acidophilic and non-acidophilic species of the *Alphaproteobacteria* (Fuerst et al., 1993; Kishimoto et al., 1995b).

The known members of *Octadecabacter*, unlike *Roseobacter*, do not produce bacteriochlorophyll *a* or pigments.

Another difference between *Octadecabacter* vs. *Roseobacter* and *Sulfitobacter* is the presence of gas vesicles. Phase contrast microscopy of cultures of *R. denitrificans* and *Ruegeria algicola* under different growth conditions at different stages of growth never reveals the presence of gas vesicles (Gosink et al., 1997). Likewise, there are no reports of closely related genera, including *Sulfitobacter*, that produce gas vesicles.

TABLE BXII.α.64. Features that differentiate *Octadecabacter* from *Roseobacter* and *Sulfitobacter*^{a,b}

Characteristic	<i>Octadecabacter</i>	<i>Roseobacter denitrificans</i>	<i>Sulfitobacter pontiacus</i>
Motility	–	+	+
Poly-β-hydroxybutyrate granule production	–	–	+
Colony color	White	Brick red	Colorless
Bacteriochlorophyll <i>a</i>	–	+	–
Growth at 19°C	–	+	+
<i>Utilization as a carbon source:</i>			
N-acetyl-glucosamine	D	–	–
Citrate	D	+	–
Fructose	–	+	–
Glycerol	D	–	+
Leucine	–	w	–
Pyruvate, propionate	D	+	+
Ribose	D	+	–
Gelatin hydrolysis	–	+	–
Oxidase	–	+	+
NO ₃ [–] reduction	–	+	–
Mol % G + C of DNA	56–57	59.6	62.1

^aSymbols: see standard definitions; w, weak reaction; blank space, not determined or not applicable.

^bData from Shiba, 1991a; Sorokin and Lysenko, 1993; Sorokin, 1995; Gosink et al., 1997.

TAXONOMIC COMMENTS

Eighteen strains of bacteria have been associated with the genus *Octadecabacter*. Of these, four strains (238, 307, 308, and 309) have been examined closely, and two strains (238 and 307) have been officially recognized (Gosink et al., 1997).

Phylogenetic analysis of the 16S rDNA of strains 238 and 307 shows that *Octadecabacter* is a member of the *Alphaproteobacteria* and that it is related to the genera *Roseobacter* (Shiba, 1991a; Gosink et al., 1997) and *Sulfitobacter* (Sorokin, 1995; Gonz  les and Moran, 1997). Several different methods were employed in analyzing the 16S rDNA sequences, including distance, parsimony, and likelihood methods, using various parameters for each type of analysis. Some of the trees obtained by these various methods differed from each other depending on which evolutionary framework (distance, parsimony, or likelihood) and what specific assumptions (transition/transversion ratio, weighting sets, step matrices, etc.) were applied. Regardless of the method employed, *Octadecabacter* is rooted deeply within the *Rhodobacter* group (Maidak et al., 1999) but not within the genera *Paracoccus* or *Rhodobacter*. The most likely tree, as evaluated under a likelihood framework by the Kishino–Hasegawa test (Kishino and Hasegawa, 1989), is shown in Fig. BXII. .78. Other phylogenetic relationships can be seen in Bowman et al. (1997a), Gonz  les and Moran (1997), and Labrenz et al. (1999).

Octadecabacter arcticus and *R. denitrificans* show DNA–DNA hybridization values of 35%   9%. *Octadecabacter antarcticus* and *R. denitrificans* have DNA–DNA hybridization values of 42%   15%. These values are below 70%, the minimum value required to be considered different isolates of the same species (Wayne et al., 1987).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *OCTADECABACTER*

Characteristics differentiating *Octadecabacter arcticus* from *Octadecabacter antarcticus* are listed in Table BXII. .65. Other characteristics are listed in Table BXII. .66.

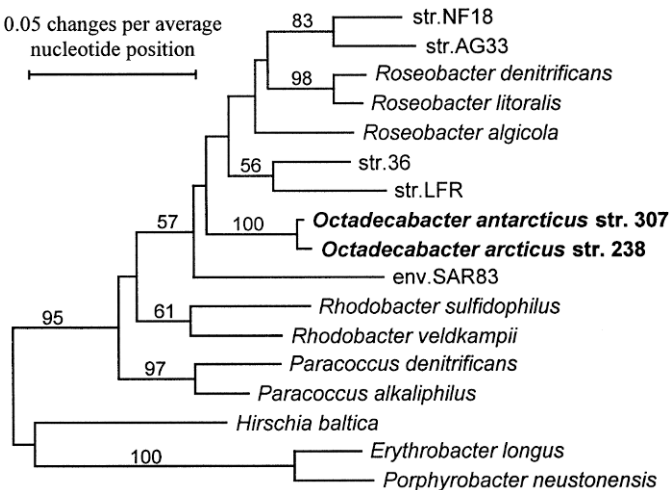


FIGURE BXII. .78. Phylogenetic position of *Octadecabacter* in relation to *Roseobacter* and closely related species. This tree is depicted under a likelihood framework with a transition to transversion ratio of 1.15. The scale bar represents approximately 0.05 changes per average nucleotide position. Numbers above the branches indicate percent bootstrap support for that branch out of 100 likelihood bootstrap resamplings. (Reprinted with permission from J.J. Gosink, et al., Systematic and Applied Microbiology, 1997, 20: 356–365.)

TABLE BXII. .65. Characteristics differentiating between the species of the genus *Octadecabacter* and two unnamed *Octadecabacter* strains^a

Characteristic	<i>Octadecabacter arcticus</i> strain 238	<i>Octadecabacter antarcticus</i> strain. 307	<i>Octadecabacter</i> sp. strain 308	<i>Octadecabacter</i> sp. strain 309
Colony color	White	White	White	White
Growth at pH values of:				
8.5	w	+	+	+
9.5	–	+	+	+
Growth at 15�C	w	–	–	–
Growth in 4.8% NaCl	w	+	+	+
Growth with carbon sources:				
N-Acetyl-glucosamine	–	w	–	+
DL-Aspartate	w	–	–	+
Citrate, D-ribose	w	–	–	–
D-Glucose, succinate	w	w	–	w
L-Glutamate	w	+	w	+
Glycerol	+	w	+	+
Glycolic acid	w	w	–	+
Propionate	w	–	+	+
Pyruvate	w	–	w	w
Requirement for vitamins:				
Biotin	–	– ^b	+	+
Thiamine, nicotinic acid, pantothenic acid	+	– ^b	+	+

^aSymbols: +, positive; –, negative; w, weakly positive.
^bA cofactor or vitamin is required other than biotin, thiamine, nicotinic acid, or pantothenic acid.

List of species of the genus *Octadecabacter*

1. ***Octadecabacter arcticus*** Gosink, Herwig and Staley 1998, 327^{VP} (Effective publication: Gosink, Herwig and Staley 1997, 363.)

arc' ti. cus. M.L. gen. *arcticus* of the arctic.

The characteristics are as described for the genus and listed in Tables BXII.α.65 and BXII.α.66, with the following additional information. Long irregular or pleomorphic rods from 0.6–0.8 × 2.4–4.0 μm. Known strains produce gas vesicles. The organisms require thiamine, nicotinic acid, and pantothenic acid for growth. Grow well on glycerol and

mixed amino acids only in the presence of small amounts of yeast extract. Grow at temperatures from 4–15°C. Predominant fatty acids when grown on Marine medium 2216 at 10°C are C_{18:1 ω7c}, C_{18:1 ω9t} or C_{18:1 ω12t} (75%), C_{16:1 ω7c} (8%), C_{16:0} (6%), and C_{10:0 3OH} (4%).

The mol% G + C of the DNA is: 57 (HPLC).

Type strain: 238.

GenBank accession number (16S rRNA): U73725.

2. ***Octadecabacter antarcticus*** Gosink, Herwig and Staley 1998, 327^{VP} (Effective publication: Gosink, Herwig and Staley 1997, 363.)

ant. arc' ti. cus. M.L. gen. *antarcticus* of Antarctica

The characteristics are as described for the genus and listed in Tables BXII.α.65 and BXII.α.66, with the following additional information. Long irregular or pleomorphic rods 0.6–0.8 × 1.6–4.8 μm. Known strains produce gas vesicles. Grow well on L-glutamate and mixed amino acids in the presence of small amounts of yeast extract. Do not grow on vitamin-free media supplemented with only biotin, thiamine, nicotinic acid, and pantothenic acid. Growth temperature range is 4–10°C. On Marine Medium 2216 at 10°C the predominant fatty acids are C_{18:1 ω7c}, C_{18:1 ω9t} or C_{18:1 ω12t} (77%), C_{16:1 ω7c} (12%), C_{16:0} (6%), and C_{10:0 3OH} (2%).

The mol% G + C of the DNA is: 56 (HPLC).

Type strain: 307, CIP 106732.

GenBank accession number (16S rRNA): U14583.

TABLE BXII.α.66. Other characteristics of the genus *Octadecabacter*^a

Characteristic	Reaction or result
Growth at pH:	
5.5	–
6.5–7.6	+
Growth at a temperature of:	
4–10 °C	+
19°C or greater	–
Salinity growth range::	
0–0.24% NaCl	–
1.2–4.8% NaCl	+
Growth with carbon sources:	
Casamino acids	+
Acetate, butyrate, ethanol, D-fructose, L-leucine, methanol, L-proline, sucrose	–
Hydrolysis of gelatin, starch	–

^aSymbols: +, positive; –, negative.

Genus XII. ***Paracoccus*** Davis 1969, 384^{AL} emend. Ludwig, Mittenhuber and Friedrich 1993, 366

ROB J.M. VAN SPANNING, ADRIAAN H. STOUTHAMER, SIMON C. BAKER AND HENK W. VAN VERSEVELD

Pa. ra. coc' cus. Gr. prep. *para* like, alongside of; Gr. n. *coccus* a grain, berry; M.L. masc. n. *Paracoccus* like a coccus.

Spherical cells 0.5–0.9 μm in diameter **or very short rod-shaped cells** 1.1–1.3 μm in diameter. **Occur singly, in pairs, or in small clusters.** Usually not encapsulated. **Gram negative.** Nonspore-forming and nonmotile. **Aerobic with a strictly respiratory metabolism.** **N-oxides** such as nitrate, nitrite, and nitrous oxide **can be used as terminal electron acceptors** for respiration under anaerobic conditions. **Nitrate is reduced to N₂** via nitrite, nitric oxide, and nitrous oxide (denitrification). Oxidase and catalase positive. **Chemoorganotrophic growth** occurs with a wide variety of organic compounds as carbon source. **Chemolithoautotrophic growth** occurs with CO₂ as carbon source, and H₂, methanol, methylamine, or thiosulfate as the electron-donating free-energy source. **Occur in water, soil, sewage, and sludge.**

The mol% G + C of the DNA is: 64–67.

Type species: ***Paracoccus denitrificans*** (Beijerinck and Minkman 1910) Davis 1969, 384 emend. Rainey, Kelly, Stackebrandt, Burghardt, Hiraishi, Kaayama and Wood 1999, 650 (*Micrococcus denitrificans* Beijerinck and Minkman 1910, 53.)

FURTHER DESCRIPTIVE INFORMATION

Paracoccus strains grow readily on ordinary media. No growth factors are required during heterotrophic growth. Colonies that are present on nutrient agar after 3 days incubation at 30°C are usually 2–4 mm in diameter, circular, smooth, low convex, moist,

and opaque. The cells do not produce carotenoid pigments. The cell wall is of type II or III (Walther-Mauruschat et al., 1977) and its composition is typical for Gram-negative bacteria. It has a thickness of 25–55 nm. No special structures have been observed in response to changes in growth conditions (Kocur et al., 1968; Sleytr and Kocur, 1973; Walther-Mauruschat et al., 1977; Nokhal and Mayer, 1979). During growth with an excess of carbon or energy source, intracellular granules of poly-β-hydroxybutyrate (PHB) are set aside as reserve for carbon and energy. PHB enhances the viability of resting cells. No extracellular hydrolysis of PHB occurs.

Quinones The presence of ubiquinone-10 is a very characteristic feature of all bacteria belonging to this group (for references see Stouthamer, 1992). In general, ubiquinone-8 is characteristic for the *Betaproteobacteria* and ubiquinone-9 for the *Gammaproteobacteria*. The nature of the respiratory quinone is therefore an important chemotaxonomic trait.

Fatty acid analyses Fatty acid methyl ester analyses of members of the genus have shown that strain LMD 22.21 and relatives cluster together, while *P. denitrificans* GB17 and strain LMD 52.44 are well separated (Baker et al., 1995). Approximately 80% of the total fatty acids is oleic acid (Wilkinson et al., 1972). The predominant phospholipid is phosphatidylglycerol (52%) The

remaining phospholipids are mostly phosphatidylcholine (31%), phosphatidylethanolamine (5.8%), and cardiolipin (3.2%). Lipid A analyses confirm not only the present taxa of the purple nonsulfur bacteria (formerly *Rhodospirillaceae*), but also the phylogenetic relatedness of distinctly phototrophic to distinctly nonphototrophic bacteria, as was suggested by cataloging of 16S rRNA. For example, lipid A in lipopolysaccharides with ester-bound C_{10:0} 3OH and the rare amide-linked C_{14:0} 3 OXO is common to the phototrophic *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* and also to *P. denitrificans* and *P. versutus* (Weckesser and Mayer, 1988).

Metabolism Members of *Paracoccus* are versatile organisms that can adapt their metabolism to the prevailing environmental conditions (Stouthamer, 1980; Van Verseveld and Stouthamer, 1992; Van Spanning et al., 1995). They are nonfermentative, and they do not produce extracellular xenobiotics or metabolites. A wide variety of organic compounds may serve as sole carbon and free-energy sources during heterotrophic growth, including D-glucose, D-fructose, sucrose, trehalose, mannose, succinate, mannitol, choline, acetate, propionate, lactate, pyruvate, malonate, butyrate, malate, citrate, gluconate, *p*-hydroxybenzoate, histidine, leucine, proline, serine, asparagine, glutamine, ethanol, propanol, butanol, glycerol, and sorbitol (Vogt, 1965; Davis et al., 1969; Pichinoty et al., 1977c). No growth occurs on xylose, rhamnose, starch, gelatin, glycogen, lactose, cellulose, benzoate, phenol, tryptophan, arginine, threonine, ethanolamine, or urea. All enzymes of the tricarboxylic acid cycle are present in *Paracoccus*. Glucose is metabolized by the Entner–Doudoroff pathway, by the hexose monophosphate pathway (Forget and Pichinoty, 1965), or by a combination of the two. The glycolytic pathway is absent (Slabas and Whatley, 1977).

During autotrophic growth, H₂, methanol, methylamine, carbon disulfide (Jordan et al., 1997), or thiosulfate (Friedrich and Mitrenga, 1981) can be used as free-energy sources. Oxidation of methanol is catalyzed by methanol dehydrogenase, a periplasmic enzyme encoded by the *mxh* gene cluster. The enzyme contains pyrroloquinoline quinone (PQQ) as a prosthetic group. Methylamine is oxidized by methylamine dehydrogenase, a periplasmic enzyme with a tryptophan tryptophyl quinone (TTQ) as prosthetic group. The enzyme is encoded by the *mau* gene cluster. The oxidation product of methanol and methylamine is formaldehyde, which is further oxidized via formate to CO₂ in the cytoplasm by formaldehyde and formate dehydrogenases. *Paracoccus* uses the Calvin cycle to fix CO₂ during autotrophic growth (Kornberg et al., 1960), and during carbon-limited mixed autotrophic/heterotrophic growth (Van Verseveld et al., 1979). Population analysis in a denitrifying methanol-fed sand filter in a municipal wastewater treatment plant revealed that 3.5% of the total cell counts corresponded to a *Paracoccus* sp., whereas in a parallel sand filter without methanol feed and no detectable denitrification only very few *Paracoccus* spp. could be detected (Neef et al., 1996). These observations indicate that autotrophic denitrifying conditions could provide the natural environmental niche for *P. denitrificans*, even though it has been difficult to grow axenic cultures under these conditions. *P. denitrificans* GB17 (formerly *Thiosphaera pantotropha*) differs from other strains by lacking the capacity to grow with methanol. However, methanol-utilizing mutants of GB17 have been isolated (Egert et al., 1993; Moir and Ferguson, 1993; Ras et al., 1995). In these GB17 mutants, methanol is oxidized by an altered ethanol dehydrogenase (Ras et al., 1995).

Two major pathways for the oxidation of thiosulfate exist in

microorganisms (Kelly et al., 1997; Friedrich, 1998). In the first pathway, called the *Paracoccus* sulfur oxidation pathway, tetrathionate is not an intermediate. In the other pathway, tetrathionate is a free intermediate in the oxidation process. The first pathway is present in *P. versutus* and *P. denitrificans*. *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* cannot oxidize thiosulfate (Imhoff and Trüper, 1992). Other *Rhodobacter* spp. and *Rhodospseudomonas palustris* can oxidize thiosulfate and possibly use the same type of enzyme as *P. denitrificans*. Recently the gene for sulfite dehydrogenase was cloned from *P. denitrificans* GB17 and was shown to be essential for thiosulfate oxidation (Wodara et al., 1997).

P. denitrificans is sensitive to potassium cyanide. Three cytochrome oxidases, *ba*₃, *cb*₃, and *aa*₃ (de Gier et al., 1994) have been detected in *P. denitrificans*. The reduction of oxygen is coupled to the translocation of protons in the *aa*₃-type (van Verseveld et al., 1981) and *ba*₃-type (Puustinen et al., 1989; Richter et al., 1994) oxidases. The proton-pumping capacity of the *cb*₃-type is still a matter of debate (de Gier et al., 1994; Raitio and Wikström, 1994).

Gases other than CO₂ produced by *Paracoccus* are nitric oxide, nitrous oxide, and dinitrogen during denitrification.

In the absence of oxygen, nitrate can serve as terminal electron acceptor for respiration. Nitrate is then reduced via nitrite, nitric oxide, and nitrous oxide, by the corresponding reductases (Boogerd et al., 1981; Timkovich et al., 1982; Snyder and Hollocher, 1987).

P. denitrificans is able to express two different types of nitrate reductase. One is membrane bound, encoded by the *narGHJ* gene cluster, and expressed during the shift from aerobic to anaerobic growth. The enzyme has its catalytic center facing the cytoplasm and has the ability to reduce chlorate as well. *In vivo*, both methyl and benzylviologens may act as electron donors to this enzyme. The other nitrate reductase is located in the periplasm, and is encoded by the *nap* operon. It is expressed independently of the oxygen concentration. This enzyme is unable to reduce chlorate. Both nitrate reductases carry molybdenum in their catalytic pocket.

Nitrite reductase is a periplasmic enzyme of the *cd*₁-type (Timkovich et al., 1982). It consists of two identical subunits of about 63 kDa, each subunit harboring one heme *c* and one heme *d*₁ (Moir et al., 1993). The X-ray structure shows that the two types of heme are located in separate domains (Fulop et al., 1993).

Nitric oxide reductase is a membrane-bound enzyme consisting of two subunits. The prosthetic groups are heme *c*, found in the small subunit, and two hemes *b* and a non-heme iron, found in the large subunit (Girsch and De Vries, 1997). With respect to its architecture and putative metal ligands, the latter subunit resembles subunit I of terminal heme-copper oxidases (Saraste and Castresana, 1994; Van der Oost et al., 1994).

Nitrous oxide reductase is a copper-containing dimeric enzyme found in the periplasm (Snyder and Hollocher, 1987). Studies on its counterpart in *Pseudomonas stutzeri* suggest that each monomer contains approximately four copper ions. Two of these form a binuclear copper center structurally similar to that of Cu_A in cytochrome *c* oxidases (Farrar et al., 1994).

The optimal pH for denitrification is between 7.0 and 8.5 and the optimal temperature is between 30 and 37°C. The rate of denitrification can be enhanced by adding a small amount of yeast extract.

P. denitrificans is susceptible to ampicillin, tetracycline, streptomycin, kanamycin, erythromycin, chloramphenicol, rifampi-

cin, and gentamicin. *P. denitrificans* is resistant to trimethoprim. Unable to fix nitrogen. There is no antigenic scheme available for *Paracoccus* species.

Paracoccus can use ammonium or nitrate as nitrogen sources. During oxidation of methylamine, the cell can use the released ammonia as nitrogen source. Two pathways serve for assimilation of ammonia in *P. denitrificans*, one with glutamate dehydrogenase operating at high ammonia concentrations, the other with glutamate-ammonia ligase and glutamate synthase at low concentrations (Mikes et al., 1991).

The distribution of metabolic systems among various selected species of the *Alphaproteobacteria* has been reviewed by Stoutamer (1992). Table BXII.α.67 is an update of the results from this publication. The division of the *Alphaproteobacteria* into subgroups is as given by Stoutamer (1992). When a property occurs only in some of the strains of a species, this is indicated in Table BXII.α.67 as "+". It is evident from Table BXII.α.67 that several pathways are very common in these bacteria. Denitrification, nitrogen fixation, hydrogenase formation, autotrophic CO₂ fixation by ribulose biphosphate carboxylase, and methylotrophy are widespread, but others are very characteristic for but not

limited to *Paracoccus*, such as heterotrophic nitrification, aerobic denitrification, and thiosulfate oxidation.

The physiological differences between *P. denitrificans* and *P. pantotrophus* are less fundamental than previously thought (Stoutamer et al., 1997). Heterotrophic nitrification was originally thought to be present only in strain GB17 but was later also detected in other *P. denitrificans* strains (Crossman et al., 1995). The key enzyme in this process, ammonia monooxygenase (AMO), has recently been purified from *P. denitrificans* (Moir et al., 1996a). The enzyme has a number of similarities with the AMO of the chemolithotrophic bacterium *Nitrosomonas europaea*. The hydroxylamine oxidase of *P. denitrificans* is completely different from that of *N. europaea* (Wehrfritz et al., 1993; Moir et al., 1996b) and donates electrons to the cytochrome *bc₁* complex. The process of heterotrophic nitrification is thus a process that differs in a large number of aspects from chemolithotrophic nitrification.

Recently the influence of carbon source on aerobic denitrification has been studied with chemostat cultures of *P. denitrificans*. The occurrence of aerobic denitrification was found to be strongly stimulated by growth with reduced substrates, e.g., bu-

TABLE BXII.α.67. Characteristics differentiating two denitrifying species of the genus *Paracoccus* from selected species of other genera of the class *Alphaproteobacteria* of the Phylum *Proteobacteria*^{a,b}

Characteristic	<i>Paracoccus denitrificans</i>	<i>Paracoccus versutus</i>	<i>Azospirillum lipoferum</i>	<i>Bradyrhizobium japonicum</i>	<i>Bradyrhizobium strain BTAi1</i>	<i>Erythrobacter longus</i>	<i>Methylobacterium extorquens</i>	<i>Methylobacterium organophilum</i>	<i>Methylosinus trichosporium</i>	<i>Nitrobacter winogradskyi</i>	<i>Oligotropha carboxidovorans</i>	<i>Rhizobium leguminosarum</i>	<i>Rhodobacter capsulatum</i>	<i>Rhodobacter sphaeroides</i>	<i>Rhodospseudomonas acidophila</i>	<i>Rhodospseudomonas palustris</i>	<i>Roseobacter denitrificans</i>	<i>Xanthobacter tagetidis</i>
Order: ^c																		
<i>Rhizobiales</i>				+	+		+	+	+	+	+	+			+	+		+
<i>Rhodospirillales</i>			+															
<i>Rhodobacterales</i>	+	+											+	+			+	
<i>Sphingomonadales</i>						+												
Family:																		
<i>Bradyrhizobiaceae</i>				+	+					+	+				+	+	+	+
<i>Hyphomicrobiaceae</i>																		
<i>Methylobacteriaceae</i>							+	+										+
<i>Methylocystaceae</i>									+									
<i>Rhizobiaceae</i>												+						
<i>Rhodobacteraceae</i>	+	+											+	+				
<i>Rhodospirillaceae</i>			+											+				
<i>Sphingomonadaceae</i>						+												
Denitrification	+	+	+	+		+	d	— ^d	d	+	—	+	b ^e	+	—	+	+	d
Photosynthesis	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—
Aerobic photosynthesis	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	+	—
Methylotrophy	+	+	+	+		—	+	+	+	—	—	+	—	—	+	—	—	+
Hydrogenase	+	—	+	+			—	—		—	+	+	+	+	+	+	—	+
Ribulose biphosphate carboxylase	+	+	+	+						+	+	+	+	+	+	+	—	+
Serine pathway	—	—				—	+	+	+	—	—		—	—	—	—	—	
Methanotrophy ^f	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
N ₂ fixation	—		+	+	+		—	—	+	—	—	+	+	+	+	+		+
Heterotrophic nitrification	+		c				—	—	c	—								
Thiosulfate oxidation	+	+											—	—	—	+		+

^aFor symbols see standard definitions.

^bData from Stoutamer (1992).

^cSee taxonomic outline.

^dSome methylobacteria reduce nitrate to nitrite.

^e*R. capsulatum* is able to reduce nitrate to nitrite and nitrous oxide to nitrogen.

^fAll methanotrophs also oxidize ammonia to nitrite.

tyrate (Sears et al., 1997), suggesting that the process is used to increase the capacity for electron transfer.

Genetic aspects The molecular genetics of *P. denitrificans* have been studied in greater detail in the last decade and these efforts have been summarized in detail in Baker et al. (1998).

The *P. denitrificans* Pd1222 genome harbors at least five copies of an insertion sequence IS1248. In addition, two copies of an element referred to as an integration region have been reported (Van Spanning et al., 1995).

In three strains of *P. denitrificans*, DSM 65, DSM 413, and DSM 415, large plasmids of molecular mass greater than 300×10^6 Da were resolved (Gerstenberg et al., 1982). *P. denitrificans* DSM 413 has three chromosomes of 1.83, 1.16, and 0.67 Mbp (Winterstein and Ludwig, 1998). The two smaller chromosomes were suggested to be linear molecules. Essential genes coding for respiratory enzymes were found distributed over all three chromosomes without any obvious clustering on any of the three molecules. The total genomic size of *P. denitrificans* of 3.66 Mbp is in agreement with an earlier estimate using a different approach (Nokhal and Schlegel, 1983). Mutations have been introduced randomly by chemical mutagenesis methods (Harms et al., 1996) or by locus-specific gene exchange techniques (Van Spanning et al., 1991). Strain Pd1222, a derivative of DSM 413, was selected from the results of a mutagenesis approach for enhanced frequencies of conjugation. This mutant had defects in two restriction modification systems and is widely used as host in gene cloning and transposon mutagenesis experiments.

ENRICHMENT AND ISOLATION PROCEDURES

The majority of *Paracoccus* strains can be enriched by culturing soil or mud samples in media containing CO₂ as carbon source and hydrogen, methanol, or methylamine as the free-energy source. The preferred electron acceptor under these growth conditions is oxygen. With nitrate, growth is very slow or absent (Vogt, 1965). Alternatively, *Paracoccus* can be enriched in anaerobic media supplemented with a heterotrophic carbon and energy source and nitrate as terminal electron acceptor, or in liquid minimal medium containing tartrate or succinate incubated under a nitrous oxide atmosphere (Pichinoty et al., 1977a, b, c). These approaches may not always be successful for the isolation of *P. denitrificans* because it is not one of the major denitrifiers in all types of soil (Gamble et al., 1977). *P. denitrificans* GB-17 can be isolated from wastewater and sulfide-rich ecosystems.

MAINTENANCE PROCEDURES

Stock cultures of *Paracoccus* strains can be maintained at room temperature in any rich agar-containing medium with Brain Heart Infusion (BHI, Difco) or Nutrient Broth (Difco) and 0.3% agar, pH 7.0. The cultures remain viable up to a year without subculturing if they are sealed with a rubber stopper or a cork that has been soaked in hot paraffin wax. Cultures survive for at least five years when frozen at -70°C in media to which 10–20% (w/v) glycerol has been added. Strains may also be preserved indefinitely by lyophilization.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

In general, cultivation of *Paracoccus denitrificans* is possible in any size of bottle or glass fermenter. The organism has been studied exhaustively during continuous cultivation in the chemostat and the retentostat (chemostat with 100% biomass retention) (Jones et al., 1977; Meijer et al., 1977; Van Verseveld and Stouthamer, 1978; Van Verseveld et al., 1979, 1986). A medium widely used

for large-scale cultivation is that described by Chang and Morris (1962). Sometimes it is necessary to add a trace elements solution (Boogerd et al., 1983). During autotrophic growth, 0.01% yeast extract and 0.05% sodium bicarbonate are added to initiate growth (Van Verseveld and Stouthamer, 1978).

DIFFERENTIATION OF THE GENUS *PARACOCCLUS* FROM OTHER GENERA

Table BXII.α.67 indicates the differential characteristics of *Paracoccus*.

TAXONOMIC COMMENTS

The type strain *Paracoccus denitrificans* ATCC 17741 (Davis et al., 1969), formerly designated *Micrococcus denitrificans*, was first isolated by Beijerinck (Beijerinck and Minkman, 1910; Nokhal and Schlegel, 1980). The following species have been regarded as close relatives of *Paracoccus denitrificans* and are placed in the same genus on the basis of 16S rRNA gene sequence analyses and DNA–DNA hybridization: *P. pantotrophus* (formerly designated *Thiosphaera pantotropha*) and *P. versutus* (formerly designated *Thiobacillus versutus*; Katayama et al., 1995). The recently isolated species *P. kocurii* (Ohara et al., 1990), *P. alcaliphilus* (Urakami et al., 1989b), *P. aminophilus* (Urakami et al., 1990b), *P. aminovorans* (Urakami et al., 1990b), *P. thiocyanatus* (Katayama et al., 1995), *P. solventivorans* (Siller et al., 1996), *P. marcusii* (Harker et al., 1998), *P. alkenifer* (Lipski et al., 1998), and *P. carotinifaciens* (Tsubokura et al., 1999) have also been placed in the genus *Paracoccus*. *P. halodenitrificans* has been moved from the genus *Paracoccus* into the genus *Halomonas* (Dobson and Franzmann, 1996).

The ϵ -type cytochrome and protein profiles were compared for a number of cultures of *P. denitrificans* obtained from a range of culture collections. The cultures fell into two groups corresponding to the two original isolates of this bacterial species. Members of one group, which included NCIMB 8944, ATCC 13543, ATCC 17741, ATCC 19367, Pd 1222, and DSM 413, were similar or identical to LMD 22.21. The second group, including DSM 65 and LMG 4218, were similar or identical to LMD 52.44 (Goodhew et al., 1996). These groupings were not compatible with the recorded history of culture deposition. Mass spectrometry and amino acid sequence comparisons showed that the cytochrome c_{550} of the LMD 52.44 culture group differed by 16% from that of the LMD 22.21 group, and yet there was only 1% difference from the cytochrome c_{550} of *Thiosphaera pantotropha*. These results suggest that consideration should be given to creation of a new species of *P. pantotrophus*, which would include *Thiosphaera pantotropha* and *P. denitrificans* LMD 52.44. At the moment, *P. denitrificans* and *T. pantotropha* are combined in the *Paracoccus* genus (Ludwig et al., 1993), since the nucleotide sequence of the 16S rRNA genes of these latter two organisms was found to be the same.

Additional strains have been isolated in independent studies. Eleven strains were isolated by (Nokhal and Schlegel, 1983) and analyzed based on 235 characteristics. Together with DSM 413 and DSM 415, these strains were placed into four subgroups with similarities ranging from 74 to 88%. This grouping was in accordance with that deduced from DNA–DNA reassociation kinetics (Auling et al., 1980).

The genus *Paracoccus* belongs to the α -subdivision of nonsulfur purple bacteria, the so-called *Alphaproteobacteria* (Woese, 1987), and encompasses 13 defined species that have been included in the Validation Lists. Members of the genus *Paracoccus* fall in the

Rhodobacter group of the *Alphaproteobacteria*. Nearest neighbors are *Rhodobacter*, *Amaricoccus*, *Octadecabacter*, *Rhodovulum*, *Roseobacter*, *Sagittula* and “*Tetracoccus*”. They share a number of properties with *Paracoccus*, including the ability to grow anaerobically with nitrate. The resemblance of the respiratory networks is striking, and most components of the pathways show structural homology to one another. Analyses of 23S and 16S rRNA gene sequences

indicated a close relationship to *Rhodobacter capsulatus* (Fox et al., 1980), suggesting that this organism and *Paracoccus denitrificans* arose from a common ancestor, after which it lost the genes encoding the photosynthetic apparatus. In addition, the amino acid sequence of cytochrome *c*₅₅₀ from *P. denitrificans* most closely resembles that of cytochrome *c*₂ from *R. sphaeroides* and *R. capsulatus* (Dickerson, 1980a).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *PARACOCCLUS*

The differential and other characteristics of the species of *Paracoccus* are indicated in Table BXII.α.68.

TABLE BXII.α.68. Differential features of the species of the genus *Paracoccus*^{a,b}

Characteristic	<i>P. denitrificans</i>	<i>P. alcatiphilus</i>	<i>P. alkenifer</i>	<i>P. aminophilus</i>	<i>P. aminovorans</i>	<i>P. carotinifaciens</i>	<i>P. kocurii</i>	<i>P. marcusii</i>	<i>P. methylolens</i>	<i>P. pantotrophus</i>	<i>P. thioyanatus</i>	<i>P. versutus</i>	<i>P. solventivorans</i>
Motility	—	—	—	—	—	+	—	—	—	—	—	+	—
Nitrate respiration	+	—	+	—	—	—	+	—	+	+	+	+	—
Vitamin requirement:													
Biotin	—	+	—	—	—	—	—	—	—	—	—	—	—
Thiamin	—	—	—	+	+	—	+	—	—	—	+	—	—
None	+	—	+	—	—	+	—	+	— ^c	+	—	+	+
Energy sources:													
Methanol	+	+	+	—	—	—	—	—	+	—	—	—	—
Formate	+	—	—	—	—	—	+	+	—	—	—	+	—
Ethanol	+	+	+	—	—	—	—	—	+	—	—	+	—
Butanol	+	—	—	—	—	—	—	—	—	—	+	—	—
Methylamine	+	+	—	+	+	—	+	—	+	—	—	+	—
Dimethylamine	+	—	—	+	+	—	+	—	—	—	—	—	—
Trimethylamine	+	—	—	+	+	—	+	—	—	—	—	—	—
Glycerol	+	+	—	+	+	+	—	+	+	—	—	+	—
Glucose	+	—	—	+	+	+	—	+	+	+	+	+	—
Fructose	+	+	—	—	+	—	—	+	+	+	+	+	—
Galactose	+	+	—	+	+	+	—	+	+	—	+	+	—
Ribose	—	—	—	+	+	—	+	—	+	—	+	—	+
Maltose	+	—	—	—	—	+	—	+	+	+	—	+	—
Sucrose	+	—	—	—	—	+	—	+	+	—	—	+	—
Lactose	—	—	—	—	—	+	—	+	—	—	—	—	—
Mannitol	+	+	—	—	+	+	—	+	+	+	+	+	—
Inositol	+	+	—	—	—	—	—	+	+	—	—	—	—
Xylose	+	+	—	+	—	+	—	—	—	—	—	—	—
Mannose	—	+	—	—	+	+	—	+	+	+	—	—	—
Sorbitol	+	+	—	—	+	+	—	+	+	+	—	—	—
Arabinose	+	+	—	+	+	+	—	+	+	—	+	+	—
Trehalose	+	—	—	—	—	+	—	+	—	—	—	—	—
Benzoate	—	—	—	—	—	—	—	—	—	+	+	—	—
Gluconate	+	—	—	—	+	+	—	+	—	+	+	+	+
Acetate	+	+	—	+	+	—	—	—	+	+	+	+	+
Lactate	—	—	—	—	—	—	—	+	+	+	+	+	—
Citrate	—	+	—	—	—	—	—	+	+	+	—	—	—
Succinate	+	+	—	+	+	—	—	+	+	+	+	+	+
Malate	+	—	—	—	—	+	—	+	+	+	—	+	—
Pyruvate	+	—	+	—	+	—	+	—	+	+	+	+	+
Trimethyl-N-oxide	—	—	—	+	+	—	+	—	—	—	—	—	—
Formamide	—	—	—	+	+	—	—	—	—	—	—	—	—
N-methylformamide	—	—	—	+	+	—	—	—	—	—	—	—	—
N,N-dimethylformamide	—	—	—	+	+	—	—	—	—	—	—	—	—
Thiosulfate	—	—	—	—	+	—	—	—	—	—	+	—	+
Mol% G + C of DNA	66.5	64.6		63.8	66.8	67	70.2	66	67	66	66.5	66.8	68.5

^aFor symbols see standard definitions.

^bRecently, three additional species of *Paracoccus* have been described: *Paracoccus kondratievae* (Doronina and Trotsenko, 2000, 2001a), *Paracoccus seriniphilus* (Pukall et al., 2003), and *Paracoccus zeaxanthinifaciens* (Berry et al., 2003).

^cRequires vitamin B₁₂ (Doronina et al., 1998b).

List of species of the genus *Paracoccus*

1. ***Paracoccus denitrificans*** (Beijerinck and Minkman 1910) Davis 1969, 384 emend. Rainey, Kelly, Stackebrandt, Burghardt, Hiraishi, Kaayama and Wood 1999, 650 (*Micrococcus denitrificans* Beijerinck and Minkman 1910, 53.)

de.ni.tri'fi.cans. L. prep. *de* away from; L. n. *nitrum* soda; M.L. *nitrum* nitrate; M.L. v. *denitrifico* denitrify; M.L. part. adj. *denitrificans* denitrifying.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are rod shaped, $0.4\text{--}0.5 \times 1.1\text{--}1.7 \mu\text{m}$, occurring singly or in pairs. Sometimes motile with a tuft of flagella. The optimal growth temperature is $30\text{--}37^\circ\text{C}$; the optimal pH for growth is 7.5–8. Electron acceptors are oxygen and nitrate. N_2 is produced from nitrate from respiration. S^0 is oxidized slowly. Tetrathionate and thiocyanate do not support growth. In addition to those mentioned in Table BXII.α.68, glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-cysteine, L-phenylalanine, L-tyrosine, L-histidine, L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-lysine, L-arginine, L-ornithine, L-cellobiose, adipate, tartrate, sarcosine, creatine, *m*-hydroxybenzoate, *p*-hydroxybenzoate, glycerate, formaldehyde, lactate, and butyrate can serve as energy and carbon sources, but cyclohexanol, L-methionine, L-tryptophan, *p*-aminobenzoate, and DL-mandelate do not. Some strains can grow with carbon disulfide as sole source of carbon and energy. Some if not all strains contain megaplastids of at least 450 kb in size. For other variations between strains, see Nokhal and Schlegel (1983), Urakami et al. (1989b), and Rainey et al. (1999).

The mol% G + C of the DNA is: 64–67 (T_m).

Type strain: ATCC 17741, DSM 65, DSM 413, LMD 22.21, LMG 4218.

GenBank accession number (16S rRNA): Y16927, Y16935, D13480, Y16928, X69159.

2. ***Paracoccus alcaliphilus*** Urakami, Tamaoka, Suzuki and Komagata 1989b, 118^{VP}

al.ca.li.phi'lus. M.L. n. *alcali* from Arabic *al* end; *qaliy* soda ash; Gr. adj. *philum* loving; M.L. adj. *alcaliphilus* liking alkaline media.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are coccoid or rod like, $0.5\text{--}0.9 \times 0.9\text{--}2.0 \mu\text{m}$ occurring singly, in pairs, or in clusters. Light-scattering PHB inclusions are evident. The preferred electron acceptor is oxygen. Nitrite is produced from nitrate, but nitrate respiration is not performed. See Table BXII.α.68 for use of energy sources. Biotin is required for growth on minimal media. Optimal growth temperature, 30°C ; optimal pH for growth, 8–9. NaCl is inhibitory at concentrations $>3\%$. Isolated from wastewater (activated sludge).

The mol% G + C of the DNA is: 64–66 (T_m).

Type strain: TK 1015, ATCC 51199, DSM 8512, JCM 7364.

GenBank accession number (16S rRNA): D32238.

3. ***Paracoccus alkenifer*** Lipski, Reichert, Reuter, Spröer and Altendorf 1998, 535^{VP}

al.ke'ni.fer. M.L. n. *alkenum* unsaturated hydrocarbons; L. suff. *-fer* carrying; M.L. adj. *alkenifer* referring to the occurrence of unusual monounsaturated fatty acids in whole-cell hydrolysates.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are coccoid or rod like, $0.4\text{--}0.6 \times 0.9\text{--}1.7 \mu\text{m}$. Nitrate is reduced to N_2 . See Table BXII.α.68 for use of energy sources. Carbohydrates and amines are not utilized. Lactate, butyrate, hydroxybutyrate, asparagine, and acetone are utilized. Optimal growth temperature, 30°C ; optimal pH for growth, 6–9. Isolated from biofilters used in treatment of waste gas from an animal rendering plant.

The mol% G + C of the DNA is: not reported.

Type strain: A901/1, DSM 11593.

GenBank accession number (16S rRNA): Y13827.

4. ***Paracoccus aminophilus*** Urakami, Araki, Oyanagi, Suzuki and Komagata 1990b, 289^{VP}

a.mi.no'phi.lus. M.L. n. *aminum* amine; Gr. adj. *phila* loving; M.L. adj. *aminophilus* amine loving.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are coccoid or rod like, $0.5\text{--}0.9 \times 0.9\text{--}2.0 \mu\text{m}$, occurring singly, in pairs, or in clusters. Light-scattering PHB inclusions are evident. The preferred electron acceptor is oxygen. Nitrite is produced from nitrate, but nitrate respiration is not performed. See Table BXII.α.68 for use of energy sources. Thiamine is required for growth on minimal media. NaCl is inhibitory at concentrations $>3\%$. Optimal growth temperature, 30°C ; optimal pH for growth, 6.5–8.0. Isolated from soil.

The mol% G + C of the DNA is: 68.5 (HPLC) or 70 (T_m).

Type strain: DM-15, ATCC 49673, DSM 8538, JCM 7686.

GenBank accession number (16S rRNA): D32239.

5. ***Paracoccus aminovorans*** Urakami, Araki, Oyanagi, Suzuki and Komagata 1990b, 289^{VP}

a.mi.no'vo.rans. L. n. *aminum* amine; L. v. *voro* to eat, devour; L. part. adj. *vorans* eating; *aminovorans* amine eating.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are coccoid or rod like, $0.5\text{--}0.9 \times 0.9\text{--}2.0 \mu\text{m}$, occurring singly, in pairs, or in clusters. Light-scattering PHB inclusions are evident. The preferred electron acceptor is oxygen. Nitrite is produced from nitrate, but nitrate respiration is not performed. See Table BXII.α.68 for use of energy sources. Thiamine is required for growth on minimal media. NaCl is inhibitory at concentrations $>3\%$. Optimal growth temperature is $30\text{--}37^\circ\text{C}$; optimal pH for growth, 7–8. Isolated from soil.

The mol% G + C of the DNA is: 67–68 (T_m).

Type strain: DM-82, ATCC 49632, DSM 8537, JCM 7685.

GenBank accession number (16S rRNA): D32240.

6. ***Paracoccus carotinifaciens*** Tsubokura, Yoneda and Mizuta 1999, 281^{VP}

ca.ro.ti.ni.fa'ci.ens. M.L. neut. n. *carotinum* carotene; L. part. adj. *faciens* making, producing; M.L. part. adj. *carotinifaciens* carotene/carotenoid-producing.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are rod shaped, $0.3\text{--}1.0 \times 1.0\text{--}5.0 \mu\text{m}$. Motile by means of peritrichous flagella. Cells are elongated or swollen at the early stage of growth with 1% yeast extract present in liquid me-

dium. Nitrate is not reduced. Produces astaxanthin. Colonies are orange to red. Optimal growth temperature, 25–30°C, no growth at 37°C; optimal pH for growth, 8–9. Isolated from soil.

The mol% G + C of the DNA is: 67 (T_m).

Type strain: E-396, IFO 16121.

7. **Paracoccus kocurii** Ohara, Katayama, Tsuzaki, Nakamoto and Kuraishi 1990, 293^{VP}
ko.cu'ri.i. M.L. gen. n. *kocurii* of Kocur; named after Miloslav Kocur, Czechoslovakian bacteriologist.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are rod shaped, 0.5–0.8 × 0.7–1.1 µm, occurring singly or in pairs. Light-scattering PHB inclusions are evident. The preferred electron acceptor is oxygen. Nitrogen is produced from nitrate. In addition to those mentioned in Table BXII.α.68, *n*-butyrate and lactate can serve as carbon and energy sources, but cyclohexanol, glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-cysteine, L-methionine, L-phenylalanine, L-tyrosine, L-histidine, L-tryptophan, L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-lysine, L-arginine, L-ornithine, L-cellobiose, adipate, tartrate, *p*-hydroxybenzoate, *p*-aminobenzoate, and DL-mandelate do not. Thiamine is required for growth on minimal media. NaCl is inhibitory in concentrations at or above 3%. Optimal growth temperature, 25–30°C; optimal pH for growth, 6.6–8.2. Isolated from wastewater (activated sludge).

The mol% G + C of the DNA is: 71 (T_m).

Type strain: B, ATCC 49631, DSM 8536, JCM 7684.

GenBank accession number (16S rRNA): D32241.

8. **Paracoccus marcusii** Harker, Hirschberg and Oren 1998, 547^{VP}
mar.cu'si.i. N.L. adj. *marcusii* referring to the late Menashe Marcus, an Israeli geneticist.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cocci to short rods, 1–2 × 1.0–1.5 µm, occurring in pairs or short chains. Colonies are bright orange, due to the accumulation of carotenoids, including astaxanthin. Grows on a wide range of carbon sources. In addition to those mentioned in Table BXII.α.68, cellobiose, lactose, melibiose, gentiobiose, glucuronic acid, galacturonic acid, erythritol, xylitol, adonitol, arabinol, propionate, *cis*-aconitate, malonate, and alanine can serve as carbon and energy sources, but fucose, rhamnose, raffinose, starch, glycogen, *N*-acetylglucosamine, *N*-acetylgalactosamine, α-glycerolphosphate, glucose-1-phosphate, glucose-6-phosphate, 2,3-butanediol, Tween 40 and 80, asparagines, aspartate, glutamate, histidine, leucine, ornithine, phenylalanine, proline, serine, threonine, inosine, uridine, and thymidine do not. Weak growth in 6% NaCl. No growth at NaCl >8%. Optimal growth temperature, 25–30°C, weak growth at 35°C; optimal pH for growth, 7. Isolated as a contaminant on agar plates.

The mol% G + C of the DNA is: 66 (HPLC).

Type strain: MH1, DSM 11574.

9. **Paracoccus methylutens** Doronina, Trotsenko, Krausova and Suzina 1998c, 1083^{VP} (Effective publication: Doronina, Trotsenko, Krausova and Suzina 1998b, 235.)
me.thyl.u'tens. M.L. neut. n. *methylum* methyl compound; L. part. adj. *utens* using; *methylutens* using methyl groups.

The description is as given for the genus with the following additional features. It is a facultative methylotrophic species capable of growth on dichloromethane, methanol, methylamine, and formate, but not formaldehyde. Nitrate is reduced to nitrite. Grows on a wide range of organic substrates, but not on acetamide, rhamnose, raffinose, or trehalose. Its major fatty acid is C_{18:1 ω7}.

The mol% G + C of the DNA is: 67 (T_m).

Type strain: DM12, VKM B-2164.

10. **Paracoccus pantotrophus** (Robertson and Kuenen 1984) Rainey, Kelly, Stackebrandt, Burghardt, Hiraishi, Katayama and Wood 1999, 650^{VP} (*Thiosphaera pantotropha* Robertson and Kuenen 1984, 91.)

pan.to'troph.us. Gr. pre. *panto* all; Gr. n. *trophos* feeder; M.L. adj. *pantotrophus* omnivorous.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cocci occurring singly, in pairs, or short chains. In addition to those mentioned in Table BXII.α.68, fumarate can be used as electron acceptor, acetoin is produced, grows on adipate, propionate, acetone, propane-1,2-diol, isopropanol, glutamate, isoleucine, leucine, alanine, histidine, proline, propanol, acetol, and propionaldehyde, and does not grow on pimelate, methylacetate, methyl ethyl ketone, propylene oxide, dulcitol, and glycogen. Autotrophic growth on sulfide, thiosulfate, and hydrogen under both aerobic and denitrifying conditions by the type strain. All three substrates can be used for mixotrophic growth. Some strains are capable of aerobic denitrification. Some strains are capable of aerobic growth on methanol and formate. Some strains contain plasmids of 85–110 kb in size and megaplasmids greater than 450 kb in size. Optimal growth temperature, 37°C (range 15–42°C); optimal pH for growth, 8 (range 6.5–10.5). Isolated from a sulfide-oxidizing, effluent treatment pilot plant.

The mol% G + C of the DNA is: 66 (T_m).

Type strain: ATCC 35512, DSM 2944, LMD 82.5.

GenBank accession number (16S rRNA): Y16933.

11. **Paracoccus solventivorans** Siller, Rainey, Stackebrandt and Winter 1996, 1129^{VP} emend. Lipski, Reichert, Reuter, Spröer and Altendorf 1998, 535.

sol.ven.ti.vo'rans. L. v. *solvo* melt, free; M.L. neut. n. *solventum* solvent; L. v. *voro* to eat, devour; L. part. adj. *vorans* eating; *solventivorans* solvent-eating.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. This organism has a Gram-negative cell wall architecture, but the Gram stain reaction is variable. Cells are coccoid or rod like, 0.4–0.5 × 0.9–1.5 µm. Light-scattering PHB inclusions are evident. NaCl is inhibitory at concentrations >0.2%. Optimal growth temperature, 30–37°C; optimal pH for growth, 7–8. No vitamin addition is required. The electron acceptors are oxygen and nitrate. N₂ is produced from nitrate from respiration. Acetone is oxidized to CO₂ and metabolized to PHB. Besides acetone and in addition to Table BXII.α.68, 2-butanone, 2-propanol, fumarate, glutamate, aspartate, asparagine, α-ketoglutarate, isoleucine, glycine, propionate, *n*-butyrate, 3-hydroxybutyrate, acetoacetate, and Casamino Acids (Difco) can serve as carbon and energy sources. Many other substrates are not utilized. Acetone is degraded via acetoacetate.

The mol% G + C of the DNA is: 68.5 (HPLC) or 70 (T_m).

Type strain: L1, DSM 6637.

GenBank accession number (16S rRNA): Y07705.

12. **Paracoccus thiocyanatus** Katayama, Hiraishi and Kuraishi 1996, 625^{VP} (Effective publication: Katayama, Hiraishi and Kuraishi 1995, 1475.)

thi.o.cy.a'na.tus. Gr. n. *thium* sulfur; Gr. n. *kyanos* dark blue; M.L. adj. *thiocyanatus* referring to the ability to use thiocyanate.

The description is as given for the genus and in Table BXII.α.68, with the following additional features. Cells are rod shaped, $0.5\text{--}0.7 \times 0.8\text{--}1.3 \mu\text{m}$, occurring singly or in pairs. Polyhedral inclusion bodies (carboxysomes) are absent in cells. Optimal growth temperature, $30\text{--}35^\circ\text{C}$; optimal pH for growth, 7.0–8.5. The addition of thiamine is required for growth in minimal media. The electron acceptors are oxygen and nitrate. N_2 is produced from nitrate from respiration. Thiocyanate is oxidized to CO_2 . S^0 is oxidized slowly. Thiosulfate is oxidized to tetrathionate by chemolithotrophically grown cells or to sulfate by thiocyanate-grown cells. Tetrathionate does not support growth. The organism is catalase and oxidase positive. Besides thiocyanate and in addition to those mentioned in Table BXII.α.68, L-alanine, L-serine, L-leucine, L-isoleucine, L-glutamate, L-proline, L-histidine, L-phenylalanine, propionate, *n*-butyrate, glutarate, *m*-hydroxybenzoate, and *p*-hydroxybenzoate serve as carbon and energy sources, but L-aspartate, L-tryptophan, L-cysteine, cellobiose, cyclohexanol, oxalate, adipate, pimelate, *o*-hydroxybenzoate, *p*-aminobenzoate, and DL-mandelate do not.

The mol% G + C of the DNA is: 66.5 (HPLC) or 67.6 (T_m).

Type strain: THI 011, IAM 12816, IFO 14569.

13. **Paracoccus versutus** (Harrison 1983) Katayama, Hiraishi and Kuraishi 1996, 625^{VP} (Effective publication: Katayama, Hiraishi and Kuraishi 1995, 1476) (*Thiobacillus versutus* Harrison 1983, 216.)

ver.su'tus. L. adj. *versutus* versatile.

Cells are rod shaped, $0.4\text{--}0.5 \times 1.1\text{--}1.7 \mu\text{m}$, occurring singly or in pairs. Nonsporeforming. Motile with a tuft of flagella. Optimal growth temperature, $30\text{--}37^\circ\text{C}$; optimal pH for growth, 7.5–8.0. No vitamin addition is required. The electron acceptors are oxygen and nitrate. N_2 is produced from nitrate from respiration. S^0 is oxidized slowly. Tetrathionate and thiocyanate do not support growth. The organism is catalase and oxidase positive. In addition to those mentioned in Table BXII.α.68, L-alanine, L-serine, L-leucine, L-isoleucine, L-aspartate, L-glutamate, L-proline, L-histidine, L-phenylalanine, propionate, *n*-butyrate, 2-oxoglutarate, glutarate, *m*-hydroxybenzoate, and *p*-hydroxybenzoate serve as carbon and energy sources, but L-tryptophan, L-cysteine, cellobiose, cyclohexanol, oxalate, pimelate, *o*-hydroxybenzoate, *p*-aminobenzoate, and DL-mandelate do not.

The mol% G + C of the DNA is: 67–68 (T_m).

Type strain: A2, ATCC 25364, CCM 2505, DSM 582, IAM 12814.

GenBank accession number (16S rRNA): Y16932.

Genus XIII. Rhodobaca Milford, Achenbach, Jung and Madigan 2001, 793^{VP} (Effective publication: Milford, Achenbach, Jung and Madigan 2000, 25)

JOHANNES F. IMHOFF

Rho.do.bac'ca. Gr. n. *rhodon* the rose; L. fem. n. *baca* berry; M.L. fem. n. *Rhodobaca* red (rose) berry.

Cells are ovoid or rod shaped, are motile by polar flagella, and divide by binary fission. **Gram negative; belong to the Alphaproteobacteria.** Phototrophically grown cells form vesicular internal photosynthetic membranes. The color of phototrophic cultures is yellow to yellow-brown, while aerobic cultures are pink to red. **Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.**

Photoheterotrophic growth occurs under anoxic conditions in the light with a variety of organic compounds as carbon and electron sources. **Chemotrophic growth occurs by aerobic respiration.** Growth factors are required.

Mesophilic bacterium with an elevated temperature optimal and best growth at alkaline pH and in the presence of low concentrations of mineral salts.

Habitats: alkaline soda lakes of low to moderate salinity.

The mol% G + C of the DNA is: 58.8.

Type species: Rhodobaca bogoriensis Milford, Achenbach, Jung and Madigan 2001, 793 (Effective publication: Milford, Achenbach, Jung and Madigan 2000, 25.)

FURTHER DESCRIPTIVE INFORMATION

In many phenotypic properties, *Rhodobaca bogoriensis* resembles *Rhodobacter* and *Rhodovulum* species. Characteristic properties

that reflect adaptation to the natural environment, African soda lakes (Lake Bogoria, Kenya), are the high pH-optimal at pH 9 and the temperature optimal at 39°C (Milford et al., 2000). No growth occurs below 30°C . Good growth is from 0–6% NaCl with an optimum from 1–2%. *Rhodobaca bogoriensis* apparently is unable to fix dinitrogen and does not grow photoautotrophically, either with hydrogen or with sulfide as electron donors. Sulfide is oxidized to extracellular elemental sulfur (Milford et al., 2000). Spectral properties of whole cells indicate the presence of only one light-harvesting pigment complex (LHI, core antenna complex) unlike *Rhodobacter* and *Rhodovulum* species, but similar to bacteria such as *Rhodospirillum rubrum*, *Rhodobium marinum*, *Rhodocista centenaria*, *Rhodovibrio sodomense*, *Rhodospira trueperi*, and *Roseospirillum parvum*. The sequence of the 16S rDNA clearly distinguishes *Rhodobaca* from *Rhodobacter* and *Rhodovulum* species but also shows the association of this bacterium with these two genera in the Alphaproteobacteria (see Fig. 3 [p. 129] in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A. Differentiating phenotypic characteristics are given in Table BXII.α.46 of the chapter on the genus *Rhodobacter*.)

ENRICHMENT AND ISOLATION PROCEDURES

Standard media and techniques for enrichment and isolation of purple nonsulfur bacteria are suitable for *Rhodobaca* species (see chapter Genus *Rhodospirillum*). Media for the enrichment of *Rhodobaca* species should have alkaline pH and contain 1–6% mineral salts.

MAINTENANCE PROCEDURES

Cultures are well preserved by standard techniques in liquid nitrogen, by lyophilization or storage at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOBACA* FROM OTHER GENERA

Characteristic properties of *Rhodobaca* are the ovoid to rod-shaped cell morphology, the presence of vesicular internal membranes, and the content of carotenoids of the spheroidene series. All these properties are shared with *Rhodovulum* and *Rhodobacter* species. A clear differentiation of *Rhodobaca* from *Rhodobacter* and *Rhodovulum* species is possible based on 16S rDNA sequences (see Fig. 3 [p. 129] in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A. Differentiating phenotypic characteristics are given in Table BXII.α.46 of the chapter on the genus *Rhodobacter*.)

List of species of the genus *Rhodobaca*

1. ***Rhodobaca bogoriensis*** Milford, Achenbach, Jung and Madigan 2001, 793^{VP} (Effective publication: Milford, Achenbach, Jung and Madigan 2000, 25.)
bo.go.ri.en'sis. M.L. fem. adj. *bogoriensis* pertaining to Lake Bogoria, a soda lake in Kenya, Africa.

Cells are ovoid to rod shaped, $0.8\text{--}1.0 \times 0.8\text{--}1.5 \mu\text{m}$. Cells are motile by means of polar flagella. Internal photosynthetic membranes appear as vesicles.

Cultures grown anaerobically in the light are yellow to yellowish brown. When grown in the presence of oxygen, the color turns to red. Absorption spectra of living cells show maxima at 376–378, 450–455, 478–480, 508–513, 590–592, 802–805, and 860–863 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series, with demethylspheroidenone as major component.

Photoheterotrophic growth occurs anaerobically in the light with a variety of organic compounds. No evidence for photoautotrophic growth with H_2 or sulfide as electron do-

nors. Chemoheterotrophic growth aerobically in the dark occurs at the full oxygen tension of air. Carbon sources supporting excellent phototrophic growth include acetate, pyruvate, malate, succinate, fumarate, butyrate, valerate, mannitol, heptanoate, xylose, glucose, fructose, and sucrose. Ammonia, aspartate, glutamate, asparagine, glutamine, and Casamino acids are used as nitrogen sources; N_2 fixation is absent. Sulfate assimilation is present. Biotin and thiamine are required as growth factors; vitamin B_{12} is growth stimulatory.

Mesophilic and alkaliphilic bacterium with optimal growth at pH 9.0 (range: 7.5–10.0), 39°C (range: $30\text{--}45^{\circ}\text{C}$), and in the presence of 1–2% NaCl.

Habitat: alkaline and saline soda lakes exposed to the light and with reduced oxygen concentrations.

The mol% G + C of the DNA is: 58.8 (T_m).

Type strain: LBB1, ATCC 700920.

GenBank accession number (16S rRNA): AF248638.

Genus XIV. *Rhodovulum* Hiraishi and Ueda 1994a, 21^{VP}

JOHANNES F. IMHOFF

Rho.do'vu.lum. Gr. n. *rhodon* rose; L. dim. n. *ovulum* small egg; M.L. neut. n. *Rhodovulum* small red egg.

Cells are ovoid to rod shaped, are motile by means of flagella or nonmotile, and divide by binary fission. **Gram negative; belong to the Alphaproteobacteria.** Phototrophically grown cells form vesicular internal photosynthetic membranes. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series. The color of phototrophic cultures is yellow-green to yellow-brown, while aerobic cultures are pink to red. Ubiquinone 10 is the major quinone. Major fatty acids are $\text{C}_{18:1}$ (predominant), $\text{C}_{18:0}$, and $\text{C}_{16:0}$.

Cells grow preferably photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth may occur with sulfide, thiosulfate, hydrogen, and ferrous iron. The final oxidation product of sulfide is sulfate. Chemotrophic growth in the dark occurs under microoxic to oxic conditions and under anoxic conditions by fermentation and oxidant-dependent metabolism. Polysaccharides, poly- β -hydroxybutyric acid, and polyphosphates may be present as storage products. Growth factors are required.

Mesophilic marine bacteria that require sodium chloride for growth.

Habitats: marine and hypersaline environments rich in organic matter, containing hydrogen sulfide, and exposed to the light.

The mol% G + C of the DNA is: 62.1–73.2.

Type species: ***Rhodovulum sulfidophilum*** (Hansen and Veldkamp 1973) Hiraishi and Ueda 1994a, 21 (*Rhodopseudomonas sulfidophila* Hansen and Veldkamp 1973, 55; *Rhodobacter sulfidophilus* Imhoff, Trüper, and Pfennig 1984, 342.)

ENRICHMENT AND ISOLATION PROCEDURES

Standard techniques and media for the isolation of phototrophic bacteria in agar dilution series and on agar plates can be applied for *Rhodovulum* species, if appropriate concentrations of salts are included (see chapter Genus *Rhodospirillum*; Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Trüper, 1992). Media containing 2–3% NaCl will be selective for *Rhodovulum* species. Under these conditions, enrichment cultures set up for purple nonsulfur bacteria will often yield *Rhodovulum* species as dominant members. Media for enrichment and isolation with organic substrates, or, for most species, autotrophic conditions with reduced sulfur compounds or hydrogen, may be chosen. Vitamins must be supplied. For *R. iodolum* and *R. robiginosum*, media with ferrous iron and containing 2.5–5% NaCl provide selective conditions.

MAINTENANCE PROCEDURES

Cultures are well preserved by standard techniques in liquid nitrogen, by lyophilization, or storage at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOVULUM* FROM OTHER GENERA

Differentiation of the genera and species of *Rhodobacter*, *Rhodobaca*, and *Rhodovulum* is possible based on 16S rDNA sequences (see Fig. 3 [p. 129] in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A, and by DNA-DNA hybridization. Differentiating phenotypic characteristics are given in Table BXII.α.46 of the chapter on the genus *Rhodobacter*.)

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOVULUM*

Characteristic properties to differentiate species of *Rhodovulum* are summarized in Tables BXII.α.69 and BXII.α.70.

List of species of the genus *Rhodovulum*

1. ***Rhodovulum sulfidophilum*** (Hansen and Veldkamp 1973) Hiraishi and Ueda 1994a, 21^{VP} (*Rhodopseudomonas sulfidophila* Hansen and Veldkamp 1973, 55; *Rhodobacter sulfidophilus* Imhoff, Trüper, and Pfennig 1984, 342.) *sul.fi.do'phi.lum*. M.L. n. *sulfidum* sulfide; Gr. adj. *philos* loving; M.L. adj. *sulfidophilum* sulfide loving.

Cells are ovoid to rod shaped, $0.6\text{--}0.9 \times 0.9\text{--}2.0 \mu\text{m}$, motile by means of polar flagella. Eventually, slime may be produced and short straight chains may be formed. Internal photosynthetic membranes appear as vesicles. Color of cell suspensions depends on the redox state of the culture and is from yellowish green through yellowish brown to dark brown and brown-red. In the presence of oxygen, cultures are red. Absorption spectra of living cells show maxima at 374–378, 451–455, 480–489, 508–512, 588–592, 800–805,

and 850–855 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series with spheroidene and hydroxy-spheroidene, which are converted to their corresponding ketocarotenoids under oxic conditions and thereby cause the color change to red.

Preferentially grow photoheterotrophically under anoxic conditions in the light using a variety of organic compounds as carbon sources and electron donors. Photoautotrophic growth occurs with sulfide, thiosulfate, and molecular hydrogen as electron donors. Sulfide and thiosulfate are oxidized to sulfate without intermediary accumulation of elemental sulfur; elemental sulfur is oxidized slowly. In mineral media sulfide is tolerated up to 5–6 mM; in the presence of complex nutrients such as 0.01% yeast extract,

TABLE BXII.α.69. Differentiating characteristics of *Rhodovulum* species^a

Characteristic	<i>R. sulfidophilum</i>	<i>R. adriaticum</i>	<i>R. euryhalinum</i>	<i>R. iodolum</i>	<i>R. robiginosum</i>	<i>R. strictum</i>
Cell diameter (μm)	0.6–0.9	0.5–0.8	0.7–1.0	0.5–0.8	0.5–0.8	0.6–1.0
Motility	+	–	+	–	–	+
NaCl optimal (%)	1–6	2.5–7.5	0.5–12	2.5–5	2.5–5	0.8–1.0
pH optimal	6.5–8.0	6.5–7.0	7.0–8.0	6.5; 7.0–7.3 ^b	6.5; 7.3–7.7 ^b	8.0–8.5
Aerobic dark growth	+	–	–	+	+	+
Sulfide tolerance	High ^c	High	High	nd	nd	High
Sulfate assimilated	+	–	–	+	+	+
Vitamins required ^d	b, n, <i>p</i> -ABA, t	b, t	b, n, <i>p</i> -ABA, t	b, n	b, n	b, <i>p</i> -ABA, t
Utilization of:						
Citrate	–	–	–	–	–	(+)
Tartrate	–	–	–	nd	nd	(+)
Mannitol	±	–	±	+	+	–
Glycerol	+	+	+	–	+	–
Ethanol	±	+	±	–	–	–
Gluconate	nd	+	nd	nd	nd	nd
Hydrogen	+	–	+	+	+	–
Thiosulfate	+	+	+	+	+	+
Ferrous iron	–	–	–	+	+	nd
Mol% G + C of the DNA	66.3–66.6 (HPLC), 68.9–73.2 (<i>T_m</i>)	64.9–66.7 (<i>T_m</i>)	62.1–68.6 (<i>T_m</i>)	66 (HPLC)	69 (HPLC)	67.3–67.7 (HPLC)

^aSymbols: +, positive in most strains; –, negative in most strains; ± variable in different strains; (+) weak growth or microaerobic growth only; nd, not determined.

^bThe first value is with ferrous iron; the second value is with acetate.

^cGood growth at concentrations $\geq 1\text{--}2 \text{ mM}$.

^db, biotin, n, niacin, *p*-ABA, *p*-aminobenzoic acid, t, thiamine.

TABLE BXII.α.70. Photosynthetic electron donors and carbon sources of *Rhodovulum* species^a

Donor/source	<i>R. sulfidophilum</i>	<i>R. adriaticum</i>	<i>R. euryhalinum</i>	<i>R. iodosum</i>	<i>R. robiginosum</i>	<i>R. strictum</i>
Formate	+	+	±	—	—	+
Acetate	+	+	+	+	+	+
Propionate	+	+	+	+	+	+
Butyrate	+	—	+	+	—	+
Valerate	+	+	nd	+	—	+
Caproate	+	+	nd	—	—	+
Caprylate	+	nd	+	—	—	—
Pelargonate	+	nd	—	nd	nd	—
Pyruvate	+	+	+	+	+	+
Lactate	+	+	+	+	+	+
Malate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Fumarate	+	+	+	+	—	+
Tartrate	—	—	—	nd	nd	(+)
Citrate	—	—	—	—	—	+
Aspartate	±	nd	+	—	—	—
Arginine	—	nd	—	nd	nd	nd
Glutamate	+	nd	±	+	+	—
Benzoate	—	—	—	—	—	—
Gluconate	±	nd	+	nd	nd	nd
Glucose	+	+	+	—	—	+
Fructose	±	—	+	—	—	+
Mannose	±	nd	nd	nd	nd	—
Mannitol	±	—	±	+	+	—
Sorbitol	±	nd	nd	nd	nd	—
Glycerol	+	+	+	—	+	—
Methanol	—	—	—	—	—	—
Ethanol	±	+	±	—	—	—
Propanol	±	—	nd	nd	nd	—
Hydrogen	+	—	nd	+	+	nd
Sulfide	+	+	+	+	+	+
Thiosulfate	+	+	+	+	+	+
Sulfur	+	+	+	+	+	nd
Ferrous iron	—	—	—	+	+	nd

^aSymbols: +, positive in most strains; —, negative in most strains; ± variable in different strains; (+) weak growth or microaerobic growth only; nd, not determined.

the tolerance is 7–8 mM. Chemotrophic growth is possible under oxic conditions in the dark. Carbon sources utilized are shown in Table BXII.α.70.

Ammonia and, in most strains, dinitrogen but not nitrate are used as nitrogen sources. Sulfate is assimilated and can serve as sole sulfur source under photoheterotrophic growth conditions and during growth with molecular hydrogen. In addition, sulfite, thiosulfate, cysteine and reduced glutathione are used as sulfur sources. Biotin, niacin, thiamine, and *p*-aminobenzoic acid are required as growth factors.

Mesophilic marine bacterium with optimal growth at pH 6.5–8.0 (with sulfide as electron donor) or at pH 5.0–7.5 (if grown with malate), 30–35°C, and 1–6% NaCl.

Habitats: marine sediments and coastal marine waters containing hydrogen sulfide and rich in organic matter.

The mol% G + C of the DNA is: 68.9–73.2 (T_m) and 66.3–66.6 (HPLC).

Type strain: Hansen W4, ATCC 35886, DSM 1374.

GenBank accession number (16S rRNA): D16423, D13475.

2. ***Rhodovulum adriaticum*** (Neutzing, Imhoff, and Trüper 1984) Hiraishi and Ueda 1994a, 22^{VP} (*Rhodopseudomonas adriatica* Neutzing, Imhoff and Trüper 1984, 503; *Rhodobacter adriaticus* Imhoff, Trüper and Pfennig 1984, 342.) *a.dri.a' ti.cum.* M.L. adj. *adriaticum* pertaining to the Adriatic Sea.

Cells are ovoid to rod shaped, 0.5–0.8 × 1.3–1.8 μm, nonmotile, often occurring in short straight chains, form capsules, and produce slime. Internal photosynthetic mem-

branes appear as vesicles. Color of cell suspensions is yellowish brown to dark brown. Absorption spectra of living cells show maxima at 374–378, 447–450, 475–480, 508–512, 588–590, 802–805, and about 869 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Photoheterotrophic growth occurs under anoxic conditions in the light with a variety of organic compounds. Photoautotrophic growth is possible with sulfide, elemental sulfur, and thiosulfate as electron donor. During oxidation of sulfide to sulfate, elemental sulfur is intermediately formed outside the cells. Molecular hydrogen is not used. Sensitive to oxygen, but microaerobic growth in the dark is possible. In the presence of ascorbate as a reductant, phototrophic growth is stimulated. Carbon sources utilized are shown in Table BXII.α.70.

Good nitrogen sources are ammonia, dinitrogen and some amino acids; growth with urea is poor. Nitrate is not assimilated but is reduced to nitrite. Sulfate is not assimilated. Growth depends on reduced sulfur sources such as sulfide, thiosulfate, cysteine, and elemental sulfur. Biotin and thiamine are required; niacin stimulates growth.

Mesophilic marine bacterium with optimal growth at pH 6.5–7.0 (pH range: 6.9–8.5), 25–30°C, and 2.5–7.5% NaCl.

Habitats: marine sediments and coastal marine waters containing hydrogen sulfide and rich in organic matter.

The mol% G + C of the DNA is: 64.9–66.7 (T_m).

Type strain: Imhoff 6II, ATCC 35885, DSM 2781.

GenBank accession number (16S rRNA): D16418, D13476.

3. **Rhodovulum euryhalinum** (Kompantseva 1989b) Hiraishi and Ueda 1994a, 22^{VP} (*Rhodobacter euryhalinus* Kompantseva 1989b, 205.)

eu.ry.ha.li'num. Gr. adj. *eury*s wide; Gr. n. *hals* salt; M.L. adj. *euryhalinum* living in a wide range of salinity.

Cells are ovoid to rod shaped, $0.7\text{--}1.0 \times 1.5\text{--}3.0 \mu\text{m}$, motile by polar flagella, divide by binary fission and often occur in short straight chains. Internal photosynthetic membranes appear as vesicles. Color of anaerobic cultures is greenish yellow, yellowish brown, to dark brown. Aerobic cultures are red. Absorption spectra of living cells show maxima at 378, 450–460, 479–486, 509–517, 592, 805, and 855 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Photoheterotrophic growth occurs under anoxic conditions in the light with a variety of organic compounds. Photoautotrophic growth is possible with sulfide, thiosulfate, and hydrogen as electron donor. During oxidation of sulfide to sulfate, elemental sulfur is intermediately formed outside the cells. Chemotrophic growth in the dark occurs with organic substrates and under autotrophic conditions with sulfide at microoxic conditions. Carbon sources utilized are shown in Table BXII.α.70. Ammonia is used as nitrogen source. Most strains do not assimilate sulfate, but depend on reduced sulfur sources; sulfide, cysteine, and cystine are used. Biotin, thiamine, niacin, and *p*-aminobenzoate are required as growth factors.

Mesophilic marine bacterium with optimal growth at pH 7.0–8.0, 25–35°C, and 0.5–12% NaCl. No growth in the absence of NaCl.

Habitats: marine sediments, coastal marine waters, and continental salt waters containing hydrogen sulfide and rich in organic matter.

The mol% G + C of the DNA is: 62.1–68.6 (T_m); type strain: 66.3 (T_m) and 65.5 (HPLC).

Type strain: Kompantseva KA-65, DSM 4868.

GenBank accession number (16S rRNA): D13479, D16426.

4. **Rhodovulum iodosum** Straub, Rainey and Widdel 1999, 734^{VP}

i.o.do'sum. Gr. adj. *iodes* violet, rusty; M.L. adj. *iodosum* indicating the formation of rusty ferric iron deposits.

Cells are ovoid to rod shaped, $0.5\text{--}0.8 \times 2.4\text{--}3.8 \mu\text{m}$, nonmotile and divide by binary fission. Color of strictly anaerobically grown cell suspensions is yellowish to brown. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series. Photoheterotrophic growth occurs under anoxic conditions in the light using a variety of organic compounds.

Photoautotrophic growth occurs with sulfide, sulfur, thiosulfate, and molecular hydrogen as electron donors. In addition, ferrous iron serves as photosynthetic electron donor. During growth on iron sulfide, ferric iron and sulfate are formed. Chemotrophic growth under oxic conditions in the dark is possible (with acetate as carbon source). Under anoxic dark conditions, no growth occurs by fermentation or with nitrate, ferric iron, DMSO, and TMAO as electron acceptors. Carbon sources utilized are shown in Table BXII.α.70.

Ammonia is used as a nitrogen source. Sulfate is not assimilated, but reduced sulfur sources are required. Thio-sulfate, elemental sulfur, sulfide, and cysteine can be used, but not sulfate and sulfite. Biotin and niacin are required as growth factors.

Mesophilic marine bacterium with optimal growth at pH 7.0–7.3 (with acetate as substrate) or pH 6.5 (pH range: 6.3–6.8, with ferrous iron as substrate), 20–25°C, and 2.5–5% NaCl (range: 2–7%).

Habitats: intertidal mud flats.

The mol% G + C of the DNA is: 66 (HPLC).

Type strain: N1, DSM 12328.

GenBank accession number (16S rRNA): Y15011.

5. **Rhodovulum robiginosum** Straub, Rainey and Widdel 1999, 734^{VP}

ro.bi.gi.no'sum. M.L. adj. *robiginosum* rusty, indicating the formation of rusty ferric iron deposits.

Cells are ovoid to rod shaped, $0.5\text{--}0.8 \times 1.6\text{--}3.2 \mu\text{m}$, nonmotile, and multiply by binary fission. Color of strictly anaerobically grown cell suspensions is yellowish to brown. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Grow photoheterotrophically under anoxic conditions in the light using a variety of organic compounds, and photoautotrophically with sulfide, sulfur, thiosulfate, and molecular hydrogen as electron donors. In addition, ferrous iron serves as photosynthetic electron donor. During growth on iron sulfide, ferric iron and sulfate are formed. Chemotrophic growth under oxic conditions in the dark is possible (with acetate as carbon source). Under anoxic dark conditions, no growth occurs by fermentation or with nitrate, ferric iron, DMSO, and TMAO as electron acceptors. Carbon sources utilized are shown in Table BXII.α.70.

Ammonia is used as a nitrogen source. Sulfate is not assimilated, but reduced sulfur sources are required. Thio-sulfate, elemental sulfur, sulfide, and cysteine can be used, but not sulfate and sulfite. Biotin, niacin, and vitamin B₁₂ are required as growth factors.

Mesophilic marine bacterium with optimal growth at pH 7.3–7.7 (with acetate as substrate) or pH 6.5 (pH range: 6.3–6.8, with ferrous iron as substrate), 25–28°C, and 2.5–5% NaCl (range: 1–7%).

Habitats: intertidal mud flats.

The mol% G + C of the DNA is: 69 (HPLC).

Type strain: N2, DSM 12329.

GenBank accession number (16S rRNA): Y15012.

6. **Rhodovulum strictum** Hiraishi and Ueda 1995, 325^{VP}

stric'tum. M.L. part. n. adj. *strictum* strict, accurate, referring to the fact that the cells require strict growth conditions.

Cells are ovoid to rod shaped, $0.6\text{--}1.0 \times 1.0\text{--}2.5 \mu\text{m}$, motile by means of polar flagella and divide by binary fission. Phototrophically grown cells contain internal photosynthetic membranes of the vesicular type. Photosynthetic cultures are yellow-green to yellow-brown, while aerobic cultures grown in the dark are pink to red. Colonies that develop aerobically on agar media in the dark are circular, convex, with entire margins, and red in color. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series.

Facultative photoheterotrophic bacteria that can grow anaerobically in the light or aerobically in the dark at full atmospheric oxygen tension. Sulfide and thiosulfate are used as electron donors for phototrophic growth, with sulfate as the final oxidation product.

Good carbon sources for phototrophic growth are formate, acetate, propionate, butyrate, lactate, pyruvate, fumarate, malate, succinate, glycolate, fructose, alanine, leu-

cine, Casamino acids, peptone, and yeast extract. Weak or slow growth occurs with valerate, caproate, tartrate, citrate, arabinose, and glucose. No or little growth occurs with caprylate, pelargonate, malonate, sucrose, galactose, mannose, adonitol, mannitol, sorbitol, glycerol, methanol, ethanol, propanol, asparagine, aspartate, or glutamate. Sulfate is assimilated as sole sulfur source. Biotin, *p*-aminobenzoic acid, and thiamine are required as growth factors.

Mesophilic, marine bacterium with optimal growth at pH 8.0–8.5 (pH range: 7.5–9.0), 30–35°C, and in the presence of 0.8–1% NaCl (range: 0.25–3.0%).

Habitat: tidal and seawater pools and similar marine environments.

The mol% G + C of the DNA is: 67.3–67.7 (HPLC).

Type strain: MB-G2, JCM 9220.

GenBank accession number (16S rRNA): D16419.

Genus XV. *Roseibium* Suzuki, Muroga, Takahama and Nishimura 2000, 2155^{VP}

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Ro.sei' bi.um. M.L. adj. *roseus* rose/pink; Gr. n. *bios* life; M.L. neut. n. *Roseibium* pink life.

Gram-negative aerobic chemoheterotrophic motile rods (0.5–0.8 × 1.0–4.0 µm for most strains). Peritrichous flagella. **Produce bacteriochlorophyll *a* aerobically. Do not grow as anaerobic phototrophs.** Catalase and oxidase positive. Produce phosphatase and nitrate reductase. Major cellular fatty acid C_{18:1}.

The mol% G + C of the DNA is: 57.6–63.4 (HPLC).

Type species: *Roseibium denhamense* Suzuki, Muroga, Takahama and Nishimura 2000, 2155.

FURTHER DESCRIPTIVE INFORMATION

Roseibium strains were isolated from marine environments including sand and surfaces of red algae (Shiba et al., 1991; Suzuki et al., 2000).

All *Roseibium* strains tested were ONPG positive and produced indole. They hydrolyzed gelatin and utilized L-aspartate, butyrate, L-glutamate, and pyruvate. They produced acid from D-fructose,

D-glucose, maltose, and D-ribose. All strains were Voges–Proskauer negative and did not produce H₂S. They were unable to utilize ethanol, glycolate, or methanol and did not produce acid from lactose or L-arabinose. None hydrolyzed Tween 80, starch, or alginate. All were streptomycin and chloramphenicol sensitive and tetracycline and penicillin resistant (Suzuki et al., 2000).

Analysis of 16S rDNA sequences placed the *Roseibium denhamense* and *R. hamelinense* type strains in the *Alphaproteobacteria* (Suzuki et al., 2000).

ENRICHMENT AND ISOLATION PROCEDURES

These bacteria were isolated from seawater, sand, and the surfaces of marine organisms from the coasts of Australia (Shiba et al., 1991; Suzuki et al., 2000). Procedures and media used are given by Shiba et al. (1991).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ROSEIBIUM*

R. denhamense utilizes acetate, fumarate, and DL-malate; *R. hamelinense* does not. *R. hamelinense* utilizes DL-lactate; *R. denhamense* does not. *R. denhamense* produces acid from D-galactose and D-

xylose; *R. hamelinense* does not. *R. hamelinense* grows in 0% NaCl; *R. denhamense* does not.

List of species of the genus *Roseibium*

1. ***Roseibium denhamense*** Suzuki, Muroga, Takahama and Nishimura 2000, 2155^{VP}
den.ha.men'se. M.L. adj. *denhamense* referring to Denham, Australia, the source of the type strain.

Description as for the genus with the following additional characteristics: utilizes acetate, fumarate, and DL-malate; produces acid from D-galactose and D-xylose; does not grow in 0% NaCl.

The mol% G + C of the DNA is: 57.6–60.4 (HPLC).

Type strain: OCh 254, JCM 19543.

GenBank accession number (16S rRNA): D85832.

2. ***Roseibium hamelinense*** Suzuki, Muroga, Takahama and Nishimura 2000, 2155^{VP}
ha.me.li.nen'se. M.L. adj. *hamelinense* referring to Hamelin Pool, Australia, the source of the type strain.

Description as for the genus with the following additional characteristics: utilizes DL-lactate and grows in 0% NaCl.

The mol% G + C of the DNA is: 59.2–63.4 (HPLC).

Type strain: OCh 368, JCM 10544.

GenBank accession number (16S rRNA): D85836.

Genus XVI. *Roseinatronobacter* Sorokin, Tourova, Kuznetsov, Bryantseva and Gorlenko 2000b, 1415^{VP} (Effective publication: Sorokin, Tourova, Kuznetsov, Bryantseva and Gorlenko 2000a, 81)

THE EDITORIAL BOARD

Ro.se.i.nat.ro.no.bac'ter. M.L. adj. *roseus* pink; M.L. n. *natron* soda; M.L. masc. n. *bacter* rod; M.L. n. *Roseinatronobacter* pink rod from soda lake.

Gram-negative, strictly aerobic, heterotrophic, nonmotile, lemon-shaped rods (0.5–0.8 × 0.8–2.2 µm); cells single or in chains. **pH optimal 10.** [Na⁺] optimal 0.4–0.6 M. **Produce bacterio-**

chlorophyll *a*. Oxidize thiosulfate to sulfate. Do not grow autotrophically.

The mol% G + C of the DNA is: 61.5 (*T_m*).

Type species: Roseinatronobacter thiooxidans Sorokin, Tourouva, Kuznetsov, Bryantseva and Gorlenko 2000b, 1415 (Effective publication: Sorokin, Tourouva, Kuznetsov, Bryantseva and Gorlenko 2000a, 82.)

FURTHER DESCRIPTIVE INFORMATION

The organism was isolated from an alkaline lake (Sorokin et al., 2000a).

Roseinatronobacter thiooxidans ALG 1 oxidizes thiosulfate to sulfate; addition of thiosulfate to cultures growing aerobically on organic substrates increases the amount of biomass produced; thus, it is able to grow lithoheterotrophically with thiosulfate. *Roseinatronobacter thiooxidans* ALG 1 also produces bacteriochlorophyll *a* but fails to grow autotrophically in the light in the absence

of oxygen. Substrates used include acetate, aspartate, benzoate, caproate, caprylate, citrate, fructose, fumarate, glucose, glutamate, glycerol, glycolate, glyoxalate, lactate, maleate, mannitol, propionate, pyruvate, sorbitol, succinate, and valerate. Nitrate can be reduced to nitrite; nitrate, nitrite, ammonium ion, and amino acids can be used as sources of nitrogen (Sorokin et al., 2000a).

Analysis of 16S rDNA sequences placed *Roseinatronobacter* in the *Alphaproteobacteria* (Sorokin et al., 2000a).

ENRICHMENT AND ISOLATION PROCEDURES

This organism was obtained from an alkaline liquid enrichment containing acetate and thiosulfate (Sorokin et al., 2000a).

List of species of the genus *Roseinatronobacter*

1. ***Roseinatronobacter thiooxidans*** Sorokin, Tourouva, Kuznetsov, Bryantseva and Gorlenko 2000b, 1415^{VP} (Effective publication: Sorokin, Tourouva, Kuznetsov, Bryantseva and Gorlenko 2000a, 82.)
thi.o.oxi.dans'. Gr. n. *thios* sulfur; M.L. part. adj. *thiooxidans* sulfur-oxidizing.

Description: same as given for the genus.

The mol% G + C of the DNA is: 61.5 (T_m).

Type strain: ALG 1, DSM 13087.

GenBank accession number (16S rRNA): AF249749.

Genus XVII. *Roseivivax* Suzuki, Muroga, Takahama and Nishimura 1999a, 632^{VP}

CHRISTOPHER RATHGEBER AND VLADIMIR V. YURKOV

Ro.se.i.vi'vax. M.L. adj. *roseus* rose colored, pink; L. adj. *vivax* living; M.L. masc. n. *Roseivivax* pink living organism.

Cells are Gram-negative, slender rods 0.5–1.0 × 1.0–5.0 μm. Motile by means of subpolar flagella. Form pink colonies when grown on agar media due to the presence of carotenoid pigments. **Grow heterotrophically and produce bacteriochlorophyll (Bchl) *a* under aerobic conditions. Reproduction occurs by binary fission. Do not grow anaerobically under illuminated conditions.** The habitat is saline lakes.

The mol% G + C of the DNA is: 59.7–64.4.

Type species: Roseivivax halodurans Suzuki, Muroga, Takahama and Nishimura 1999a, 632.

FURTHER DESCRIPTIVE INFORMATION

Two species of *Roseivivax* are presently described, *Roseivivax halodurans* and *Roseivivax halotolerans*. Phylogenetically these species belong to the *Alphaproteobacteria*, and are most closely related to members of the genera *Roseobacter*, *Sagittula*, *Octadecabacter*, and *Sulfitobacter*. Both species form circular, smooth, slightly convex, entire, glistening, opaque, pink colonies on agar media. Although all isolated strains produce photosynthetic pigments, they grow only under aerobic conditions, and do not grow anaerobically even in the presence of light. This peculiarity is common to all representatives of the so-called aerobic phototrophic bacteria.

Cells are slender rods 0.5–1.0 × 1.0–5.0 μm, and reproduce by binary fission. Cells are motile by means of subpolar flagella.

Optimal growth occurs at pH between 7.5 and 8.0, and at temperatures between 27°C and 30°C. *Roseivivax* species show a broad tolerance for saline conditions, with growth occurring between 0% and 20% NaCl. A variety of organic substrates can be utilized for growth, although the specific substrates utilized vary between species. Members of this genus are catalase and oxidase positive, produce indole, but do not produce H₂S. Voges–Pros-

kauer test is negative, ONPG reaction is positive.

The major cellular fatty acid present is C_{18:1} and the major quinone is ubiquinone Q-10, with smaller amounts of ubiquinones Q-7, Q-8, and Q-9.

ENRICHMENT AND ISOLATION PROCEDURES

Both *R. halodurans* and *R. halotolerans* were isolated from the saline lake, Lake Clifton, on the west coast of Australia. *R. halodurans* was isolated from a sample containing charophytes, whereas *R. halotolerans* was isolated from a sample containing epiphytes, which had developed on living stromatolites. Strains were grown on PPES-II medium (Suzuki et al., 1999a) and pure cultures were obtained by replating of separate colonies.

MAINTENANCE PROCEDURES

Both *Roseivivax* spp. can be maintained on PPES-II slant agar culture medium (Shiba et al., 1991).

DIFFERENTIATION OF THE GENUS *ROSEIVIVAX* FROM OTHER GENERA

Genus *Roseivivax* can be differentiated from other known genera based on 16S rRNA gene sequence analysis, as the two known members form a distinct cluster within the *Alphaproteobacteria*. Members of this genus are related to members of the aerobic phototrophic genus *Roseobacter*, but can be differentiated from them by differences in absorption spectrum characteristics. *Roseivivax* shows an absorption spectrum similar to that of *Erythrobacter longus* (Nishimura et al., 1994), with a major peak at 871–873 nm and a smaller peak at 803–805 nm, which correspond to Bchl *a* incorporated into light-harvesting complex I and reaction centers, respectively. In contrast, the absorption spectrum of *Roseobacter* shows a major peak at about 806 nm due to the

presence of an unusual bacteriochlorophyll-protein complex, B806, as well as a smaller peak at about 870 nm, due to the light-harvesting complex I.

Roseivivax forms circular pink colonies when grown on agar media. Growth occurs heterotrophically under aerobic conditions, but not under anaerobic conditions even in the light. This peculiarity differentiates *Roseivivax* from all known genera of the purple nonsulfur bacteria, which possess Bchl *a* and can grow under both conditions, aerobic dark and anaerobic light.

Members of the genus *Roseivivax* can be differentiated from each other based on differences in 16S rRNA gene sequence, homology of the DNA, and absorption spectrum characteristics, as well as physiological and biochemical properties, including salt requirement, carbon sources utilized, presence of urease, phosphatase, gelatinase, and the ability to reduce nitrate (Table BXII.α.71).

TAXONOMIC COMMENTS

Shiba et al. (1991) described several strains of aerobic bacteriochlorophyll *a*-containing bacteria isolated from different saline locations on the east and west coasts of Australia. These strains

were divided into four groups (GI, GII, GIII, and GIV), based on cell color, type of absorption spectrum, and cell morphology. *R. halotolerans* and *R. halodurans* were placed into the GII group along with 17 other similar strains.

However, based on DNA-DNA hybridization studies, this group was further divided into four subgroups based on DNA homology. *R. halotolerans* and *R. halodurans* fell outside of these four subgroups, indicating that the genus *Roseivivax* is not closely related to any of the other strains in the GII group (Nishimura et al., 1994).

16S rRNA gene sequence analysis shows that *Roseivivax* forms a separate cluster within the *Alphaproteobacteria*. The closest relatives are members of the genera *Roseobacter*, *Sagittula*, *Octadecabacter*, and *Sulfotobacter*.

FURTHER READING

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List of species of the genus *Roseivivax*

1. ***Roseivivax halodurans*** Suzuki, Muroga, Takahama and Nishimura 1999a, 632^{VP}
ha.lo.du'rans. Gr. n. *hals* salt; L. pres. part. *durans* enduring; M.L. part. adj. *halodurans* salt enduring.

Gram-negative rods, 0.5–1.0 × 1.0–5.0 μm, motile by subpolar flagella. Form circular pink colonies on solid media. Cells contain Bchl *a* organized into reaction center and light harvesting complexes, giving absorption spectrum peaks at 803 and 873 nm, respectively. Tolerate high salt concentrations with growth occurring from 0 to 20.0% NaCl. Obligately aerobic heterotrophs, do not grow anaerobically under illuminated conditions. Cells utilize D-glucose, L-arabinose, D-fructose, D-galactose, lactose, maltose, D-ribose, sucrose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate, and L-glutamate as the sole carbon source. Cells do not utilize fumarate, glycolate, ethanol, methanol, or D-xylose. Do not hydrolyze gelatin, Tween 80, starch, or alginate. Produce catalase, oxidase, phosphatase, and nitrate reductase; do not produce urease. Voges-Proskauer test is negative, ONPG reaction is positive, produce indole but not H₂S.

Optimal growth occurs at pH 7.5–8.0 and at temperatures between 27 and 30°C. Resistant to penicillin and tetracycline, but susceptible to chloramphenicol and streptomycin.

Optimal growth occurs at pH 7.5–8.0 and at temperatures between 27 and 30°C. Resistant to penicillin and tetracycline, but susceptible to chloramphenicol and streptomycin.

TABLE BXII.α.71. Distinguishing characteristics of species *Roseivivax halodurans*, *Roseivivax halotolerans*, and the species *Rubrimonas cliftonensis*^a

Characteristic	<i>Roseivivax halodurans</i>	<i>Roseivivax halotolerans</i>	<i>Rubrimonas cliftonensis</i>
Nitrate reductase	+	–	+
Phosphatase	+	–	+
Urease	–	+	–
Hydrolysis of gelatin	–	+	–
Utilization of:			
Acetate	+	+	–
Butyrate	+	+	W
Glycolate	–	–	+
DL-Lactate	+	+	–
DL-Malate	+	+	–
Succinate	+	+	–
Ethanol	–	–	+
D-Fructose	+	W	+
D-Glucose	+	W	+
D-Galactose	+	–	+
Maltose	+	–	W
D-Ribose	+	–	+
D-Xylose	–	W	+
Lactose	+	–	–
Sucrose	+	–	–
Growth in presence of:			
0% NaCl	W	+	–
20% NaCl	W	+	–
Near infrared Bchl <i>a</i> peaks	803, 873	805, 871	806, 871

^aSymbols: +, positive; –, negative; W, weak.

tomycin. Habitat: charophytes in a saline lake, Lake Clifton, on the west coast of Australia.

The mol% G + C of the DNA is: 64.4 (HPLC).

Type strain: Och 239, ATCC 700843, CIP 105983, JCM 10272, NBRC 16685.

GenBank accession number (16S rRNA): D85829.

2. **Roseivivax halotolerans** Suzuki, Muroga, Takahama and Nishimura 1999a, 633^{VP}
ha.lo.to'le.rans. Gr. n. *hals* salt; L. pres. part. *tolerans* tolerating; M.L. part. adj. *halotolerans* salt tolerating.

Gram-negative rods, 0.5–1.0 × 1.0–5.0 µm, motile by subpolar flagella, form circular pink colonies on agar media. Cells contain Bchl *a* organized into reaction center and light harvesting complexes, exhibiting absorption spectrum peaks at 805 and 871 nm, respectively. Require salt for growth, able to tolerate high salinity with growth occurring between 0.5 and 20.0% NaCl. Aerobic heterotrophs, do not grow anaerobically under illuminated conditions. Sub-

strates utilized as sole carbon source are D-glucose, D-fructose, D-xylose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate, and L-glutamate. Does not grow on fumarate, glycolate, ethanol, methanol, L-arabinose, D-galactose, lactose, maltose, D-ribose, or sucrose. Hydrolyze gelatin, do not hydrolyze Tween 80, starch, or alginate. Produce catalase, oxidase, and urease. Do not have phosphatase or nitrate reductase activity. Produce indole but not H₂S, Voges–Proskauer test is negative, ONPG reaction is positive.

Optimal growth occurs at pH 7.5–8.0, and temperature 27–30°C. Resistant to penicillin and tetracycline, but susceptible to chloramphenicol and streptomycin. Habitat: epiphytes growing on living stromatolites in Lake Clifton, a saline lake on the west coast of Australia.

The mol% G + C of the DNA is: 59.7 (HPLC).

Type strain: Och 210, ATCC 700842, CIP 105984, JCM 10271, NBRC 16686.

GenBank accession number (16S rRNA): D85831.

Genus XVIII. *Roseobacter* Shiba 1991b, 331^{VP} (Effective publication: Shiba 1991a, 144)

TSUNEO SHIBA AND JOHANNES F. IMHOFF

Ro.se.o.bac'ter. M.L. adj. *roseus* rose-colored, pink; M.L. masc. n. *bacterequivalent* of Gr. neut. n. *bacterion* a rod; M.L. masc. n. *Roseobacter* pink rod-shaped bacterium.

Cells are ovoid or rod shaped, motile by subpolar flagella, divide by binary fission. They are **Gram negative** and are members of the **Alphaproteobacteria**. Colonies are circular, smooth, slightly convex, and pink-to-red in color. Absorption spectra of cell suspensions have major maxima in the near infrared region at 805–807 nm and a smaller one at 868–873 nm (Fig. BXII.α.79). **Bacteriochlorophyll (BChl) *a*** is present. The major carotenoid is spheroidenone. Phosphatidylglycerol and diphosphatidylglycerol are present. The main cellular fatty acid is C_{18:1}.

Aerobic chemoorganotrophic bacteria. Do not grow photo-trophically and do not produce photosynthetic pigments under anoxic conditions in the light. Carbon sources and electron donors supporting growth are simple organic compounds. Gelatin and Tween 80 are hydrolyzed. Catalase and oxidase are present. Biotin, thiamine and nicotinic acid are required as growth factors.

The major respiratory quinone is ubiquinone-10.

Mesophilic and neutrophilic bacteria from the marine environment. Optimal growth is at pH 7.0–8.0 and 20–30°C. Low concentrations of sodium ions are required.

The mol% G + C of the DNA is: 56–60.

Type species: ***Roseobacter litoralis*** Shiba 1991b, 331 (Effective publication: Shiba 1991a, 144.)

FURTHER DESCRIPTIVE INFORMATION

Roseobacter is an aerobic chemoheterotrophic bacterium containing bacteriochlorophyll (Shiba and Harashima, 1986). According to 16S rDNA sequence similarity, it is closely related to *Sulfobacter*, *Staleyia*, *Sagittula*, *Octadecabacter*, *Ruegeria*, *Antarctobacter*, *Roseovarius*, *Roseivivax*, and *Marinosulfonomonas*, which form a cluster of bacteria in the *Alphaproteobacteria* that have been isolated from marine environments. Of these bacteria only *Roseovarius*, *Roseivivax*, and *Staleyia* contain BChl *a*.

The requirement of oxygen for photosynthesis and BChl synthesis in *R. denitrificans* is in contrast to the inhibitory effect of oxygen on photosynthesis and bacteriochlorophyll synthesis in

anoxygenic phototrophic purple bacteria (Harashima et al., 1982; Shiba, 1984, 1987; Okamura et al., 1985; Nishimura et al., 1996; Porra et al., 1996; Kortlüke et al., 1997).

BChl synthesis in *Roseobacter* requires oxygen and dark conditions (Shioi and Doi, 1988; Takamiya et al., 1992). Even though the bacteria grow anaerobically with nitrate or trimethylamine-N-oxides (TMAO) as terminal electron acceptor, BChl is not synthesized under these conditions (Arata et al., 1988). The 13'-oxo group of the isocyclic ring E of BChl is derived from molecular oxygen via an oxygenase, whereas the same biosynthetic step in *Rhodobacter sphaeroides* is an anaerobic process (Porra et al., 1996).

The photosynthetic apparatus of *Roseobacter* is similar to that found in the anaerobic phototrophic bacteria of the *Rhodobacteraceae*. In *R. denitrificans*, BChl *a* is esterified with phytol as in

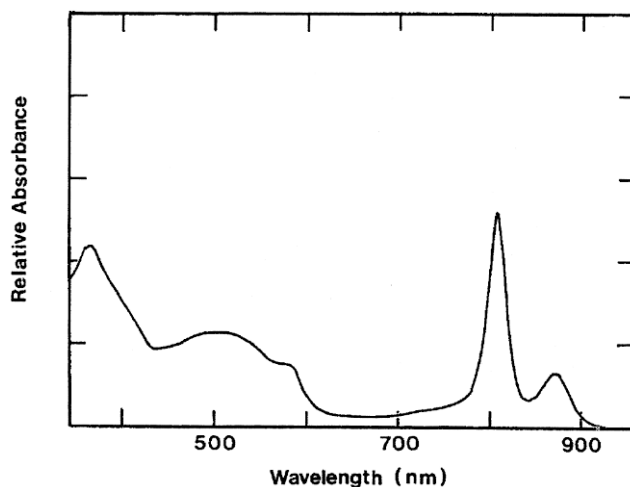


FIGURE BXII.α.79. Absorption spectrum of a membrane fraction of *Roseobacter litoralis* (Reproduced with permission from T. Shiba, *Systematic and Applied Microbiology* 14: 140–145, 1991, ©Urban & Fischer Verlag.)

Rhodobacter and many anoxygenic phototrophic purple bacteria (Shiba, 1991a). The major carotenoid is spheroidenone, an oxo-derivative of spheroidene present in *Rhodobacter* species (Harashima and Nakada, 1983). Two BChl-protein complexes, RC-870 and B806, have been isolated from *R. denitrificans* (Shimada et al., 1985). RC-870 corresponds to a reaction center-light harvesting complex (RC-LHI complex) of the anoxygenic phototrophic purple bacteria with which the complex purified from *R. denitrificans* shares almost identical absorption spectra. One mole of the RC contains 4 mol of BChl *a*, 2 mol of bacterio-phytyl *a*, 4 mol of cytochrome *c*₅₅₄, 2 mol of ubiquinone-10 and an unknown amount of carotenoids. The B806 complex corresponds to the B800-850 (LH2) of anoxygenic phototrophic purple bacteria, but lacks the absorption maximum at approx. 850 nm. The BChl-proteins of *R. denitrificans* seem to be integrated in vesicular internal membrane structures. The diameter of the vesicles, 60–120 nm, is larger than that of the anaerobic photosynthetic bacteria (40–60 nm) (Iba et al., 1988). The outer layer of *R. denitrificans* is characterized by a lipopolysaccharide containing no heptose, as in *Rhodobacter* (Neumann et al., 1995).

Roseobacter spp. are aerobic chemoheterotrophic bacteria that are unable to grow phototrophically under anoxic conditions. Light is utilized by *R. denitrificans* as an auxiliary energy source for ATP synthesis only under oxic conditions (Shiba, 1984; Okamura et al., 1985). A photosynthetic electron transfer system operates under anaerobic conditions, if the bacteria can utilize nitrate, nitrite, or TMAO as the terminal oxidant (Arata et al., 1988; Takamiya et al., 1988). Since this photosynthetic system competes with the respiratory system for the same electron transfer component, light suppresses the respiratory activity (Harashima et al., 1982). Therefore, there is no difference in exponential growth in the light and in the dark. A positive effect of light on growth is observed only when respiratory activity is suppressed under certain environmental conditions. For example, growth yield is enhanced only when cultures are illuminated at stationary growth phase (Shioi, 1986; Harashima et al., 1987). The viability of *R. denitrificans* is also enhanced by light in starvation survival media lacking energy-yielding substrates (Shiba, 1984).

Roseobacter has been isolated from green seaweed (Shiba et al., 1979; Shiba, 1991a). Although *Roseobacter* species have not been isolated from seawater, a rDNA clone SAR83, which is phylogenetically closely related to *Roseobacter denitrificans*, is predominant in the bacterial plankton population of Sargasso Sea (Britschgi and Giovannoni, 1991; Fuhrman et al., 1993). Many aerobic bacteriochlorophyll-containing bacteria, the relationship of which to *Roseobacter* is not yet clarified, have been isolated from cyanobacterial patches, seaweeds, sea grasses, sea sands, and surface seawater. The population of aerobic bacteriochlorophyll-containing bacteria may comprise as much as 20–50% of the total aerobic chemoheterotrophic bacteria (Shiba et al., 1991; Shiba, 1995).

ENRICHMENT AND ISOLATION PROCEDURES

Roseobacter strains have been isolated on agar plates with a modified PPES-II medium (Shiba, 1989) which contains per liter: 2 g polypeptone (Nihon Pharmaceutical Co.), 1 g proteose-peptone no. 3 (Difco), 1 g Bacto soytone (Difco), 1 g Bacto yeast extract (Difco), 0.1 g ferric citrate, 700 ml artificial seawater (Lyman and Fleming, 1940), and 300 ml distilled water. Inclusion of iron strongly enhances BChl synthesis. The pH is adjusted to 7.6–7.8. The agar plates are incubated at 20–25°C. Pink- or red-pigmented colonies are formed on agar plates.

Shioi (1986) used a medium which contains in 1 liter of distilled water: 20 g NaCl, 5.0 g MgCl₂·6H₂O, 2.0 g Na₂SO₄, 0.5 g KCl, 0.5 g CaCl₂·2H₂O, 0.2 g NaHCO₃, 0.1 g ferric citrate, 2.0 g yeast extract (Difco), 1.0 g polypeptone, 1.0 g Casamino acids, and 1.0 ml glycerol. The growth yield in this medium is approximately twice as high as that in PPES-II medium. Elimination of the trace elements Br, Sr, B, and F from the medium of Shioi (1986) has no effect on growth.

MAINTENANCE PROCEDURES

Roseobacter can be maintained at 20°C as stab cultures in media containing 0.4% agar (Difco). Cultures should be transferred monthly. Maintenance on agar slant cultures at room temperature is not recommended. The bacteria remain viable for 6 months when slant cultures are kept at –80°C. Cultures may also be preserved in liquid nitrogen or by lyophilization.

DIFFERENTIATION OF THE GENUS *ROSEOBACTER* FROM OTHER GENERA

Roseobacter differs from anoxygenic phototrophic bacteria in the *Rhodobacteraceae* in its inability to perform photosynthesis under anoxic conditions. Based on 16S rDNA sequence analysis, the genus is most closely related to *Sulfitobacter*, *Staleyia*, *Sagittula*, *Octadecabacter*, *Ruegeria*, *Antarctobacter*, *Roseovarius*, *Roseivivax*, and *Marinosulfonomonas* (Sorokin, 1995; González et al., 1997b; Gosink et al., 1997; Holmes et al., 1997; Labrenz et al., 1998, 1999; Uchino et al., 1998; Pukall et al., 1999; Suzuki et al., 1999a; Labrenz et al., 2000). It can be differentiated from these bacteria by pigment and phospholipid composition, capabilities for degradation of organic substances, and other properties. Unlike *Roseobacter*, cells of *Sulfitobacter*, *Sagittula*, *Octadecabacter*, *Ruegeria*, and *Antarctobacter* do not contain bacteriochlorophyll. Although *Staleyia*, *Roseivivax*, and *Roseovarius* contain BChl *a*, their absorption spectra do not show the large peak at ~800 nm. Phosphatidylethanolamine is present in the cells of *Staleyia* and *Roseovarius* but not in *Roseobacter*. Tween 80 is hydrolyzed by *Roseobacter*, *Staleyia*, and *Roseovarius tolerans* strain EL-52, but not by species of the other genera. *Staleyia*, *Roseovarius*, and *Antarctobacter* can grow at 150% seawater but *Roseobacter* cannot. *Roseobacter* differs from *Marinosulfonomonas* and *Sagittula* in lacking the capacity for methylotrophic growth (Holmes, et al., 1997). *Roseobacter* can grow at 30°C but the gas-vacuolated bacterium *Octadecabacter* cannot (Gosink et al., 1997). Differential characteristics of the genus *Roseobacter* and related genera are shown in Table BXII.α.72.

TAXONOMIC COMMENTS

The genus *Roseobacter* was proposed for aerobic chemoheterotrophic bacteria which contain BChl *a* and have a strict requirement of oxygen for synthesis of bacteriochlorophyll (Shiba, 1991a).

Subsequently, *Roseobacter algicola* and *Roseobacter gallaeciensis* were included in the genus *Roseobacter* based only on 16S rDNA sequence information, although their phenotypic characteristics were not coincident with the definition of the genus *Roseobacter* (Lafay et al., 1995; Ruiz-Ponte et al., 1998). Neither species contains BChl *a*. Data on levels of DNA–DNA reassociation to the type species of *Roseobacter litoralis* are lacking (Lafay et al., 1995). In addition, the phylogenetic position of *Roseobacter algicola* is more distant from *Roseobacter litoralis* and *R. denitrificans* than are *Sagittula*, *Sulfitobacter*, and *Octadecabacter* (González et al., 1997b; Labrenz et al., 1999).

Roseobacter algicola has been reassigned to a new genus as *Rue-*

TABLE BXII.α.72. Comparison of different genera of bacteria related to *Roseobacter*^a

	<i>Roseobacter</i>	<i>Roseobacter gallaeciensis</i>	<i>Ruegeria</i>	<i>Roseirivax</i>	<i>Roseovarius</i>	<i>Antarctobacter</i>	<i>Octadecabacter</i>	<i>Sulfobacter</i>	<i>Staleya</i>	<i>Sagittula</i>	<i>Marinosulfonomonas</i>
Bacteriochlorophyll	+	—	—	+	+	—	—	—	V	—	—
Bchl protein (nm) ^b	805/873	—	—	873	877–879	—	—	—	800-2/861-2	—	—
Motility	+	+	V	+	+	V	—	+	+	+ ^c	—
Degradation of:											
Tween 80	+	—	—	—	V	—	nd	nd	+	—	nd
Gelatin	+	—	+	V	—	+	—	V	—	—	nd
Requirement of:											
Biotin	+	—	+	nd	W	W	V	nd	W	enhance	nd
Niacin	+	—	—	nd	W	+	V	nd	W	enhance	nd
Oxidase	+	+	+	+	W	+	—	+	+	+	+
Diphosphatidylglycerol	+	nd	+	nd	+	—	nd	+	—	nd	nd
Phosphatidylethanolamine	—	nd	+	nd	+	—	nd	+	+	nd	nd
Gas vacuole	—	nd	—	nd	nd	—	+	—	+	—	—
Growth at 150% seawater	—	—	—	nd	+	+	—	—	+	—	—
Growth at:											
30°C	+	+	+	+	+	+	—	+	+	+	+
4°C	+	—	—	nd	+	+	+	+	+	+	—
Utilization of:											
Methanol	—	nd	—	—	—	—	—	—	—	+	+
Butyrate	—	+	V	+	+	+	—	+	—	+	nd

^aSymbols: +, positive; —, negative; nd, not described; V, variable; W, weak; enhance, growth enhancement.

^bCell suspension spectra.

^cMotility of *Sagittula* was assumed by the presence of flagella in the suspension, but motile cells were not seen.

geria algicola (Uchino et al., 1998) and according to 16S rDNA analysis constitutes a robust monophyletic cluster with *R. gallae-*

ciensis (Suzuki et al., 1999a). Neither species is considered in this chapter.

List of species of the genus *Roseobacter*

1. ***Roseobacter litoralis*** Shiba 1991b, 331^{VP} (Effective publication: Shiba 1991a, 144.)

li.to.ra'lis. L. gen. n. *litoris* of the seashore; M.L. masc. adj. *litoralis* belonging or pertaining to the seashore.

Cells are rod-to-ovoid shaped, 0.6–0.9 × 1.2–2.0 µm, motile by subpolar flagella. Cell suspensions and colonies are pink. Cell suspensions show a major absorption band at 805–807 nm and a minor one at 868–873 nm. Bacteriochlorophyll *a* esterified with phytol is present. The major carotenoid is spheroidenone. Phosphatidylglycerol and diphosphatidylglycerol are present. The main cellular fatty acid is C_{18:1}.

Strictly aerobic chemoheterotrophic bacteria that grow on several organic substrates under oxic conditions. They produce photosynthetic pigments under aerobic conditions, but are unable to grow photosynthetically in the absence of oxygen. Under oxic conditions, photosynthetic energy generation is possible. Methanol is not utilized. Dissimilatory nitrate reducing activity is absent. Chemoautotrophic growth with H₂ is not possible. Biotin, thiamin, and nicotinic acid are required as growth factors.

Optimal growth is at pH 7–8 and 20–30°C. Sodium ions are required.

Habitat: surface of high-tidal seaweeds.

The mol% G + C of the DNA is: 57.2 ± 0.9 (HPLC).

Type strain: OCh149, ATCC 49566, DSM 6996, IFO 15278.

GenBank accession number (16S rRNA): X78312.

2. ***Roseobacter denitrificans*** Shiba 1991a, 331^{VP} (Effective publication: Shiba 1991a, 144.)

de.ni.tri'fi.cans. M.L. part. adj. *denitrificans* denitrifying.

Cells are rod-to-ovoid shaped, 0.6–0.9 × 1.0–2.0 µm, motile by subpolar flagella. Internal photosynthetic membranes are of vesicular type. Cell suspensions and colonies are pink. Cell suspensions show a major absorption band at 805–807 nm and a minor one at 868–873 nm. Bacteriochlorophyll *a* esterified with phytol is present. The major carotenoid is spheroidenone. The major quinone is ubiquinone-10. Phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylcholine are present. The main cellular fatty acid is C_{18:1}.

Growth occurs chemoheterotrophically under oxic conditions with several organic substrates. They produce photosynthetic pigments under aerobic conditions, but are incapable of photosynthetic growth in the absence of oxygen. Under oxic conditions, photosynthetic energy generation is possible. Methanol is not utilized. Photosynthetic activity is also found in the presence of nitrate or trimethylamine-N-oxide as electron acceptors. Nitrate is reduced to N₂O.

Chemoautotrophic growth with H_2 is not possible. Biotin, thiamin, and nicotinic acid are required as growth factors.

Optimal growth is at pH 7–8 and 20–30°C. Sodium ions are required.

Habitat: surface of high-tidal seaweeds.

The mol% G + C of the DNA is: 59.6 ± 0.5 (HPLC).

Type strain: OCh114, ATCC 33942, DSM 7001, IFO 15277.

GenBank accession number (16S rRNA): L01784, M59063.

Genus XIX. *Roseovarius* Labrenz, Collins, Lawson, Tindall, Schumann and Hirsch 1999, 145^{VP}

MATTHIAS LABRENZ AND PETER HIRSCH

Ro' se.o.va' ri.us. L. adj. *roseus* rosy; L. adj. *varius* diverse, varied; M.L. masc. n. *Roseovarius* a variably rosy bacterium.

Rods, one or both cell poles pointed, multiplying by monopolar growth, i.e., by a budding process. Gram negative. Daughter cells may be motile. No resting stages are known. **Aerobic**, with a strictly respiratory type of metabolism. **Intracellular granules of poly- β -hydroxybutyrate (PHB) are present. Bacteriochlorophyll *a* (bchl *a*) may be produced**. Cells have an absolute requirement for Na^+ . Temperature range for growth <3–43.5°C, salinity range for growth <10 to >150‰ of artificial seawater (ASW), NaCl tolerance range <1.0 to 10.0% and pH range for growth 5.3 to >9. **Catalase and oxidase weakly positive**. Do not grow photoautotrophically with H_2/CO_2 (80:20) or photoorganotrophically with acetate or glutamate. Occur in marine habitats.

The mol% G + C of the DNA is: 62–64.

Type species: *Roseovarius tolerans* Labrenz, Collins, Lawson, Tindall, Schumann and Hirsch 1999, 145.

FURTHER DESCRIPTIVE INFORMATION

Visible growth of *R. tolerans* appears at 20°C after 3–5 d on medium PYGV (Staley, 1968) prepared with 25‰ (or 40‰) artificial seawater (ASW; Lyman and Fleming, 1940) or on R2A agar (Difco, Detroit) with the appropriate ASW concentration. Colonies are circular, smooth, convex, 1–2 mm in diameter and red, pink, beige to light red, beige, or whitish beige. Cell sizes: $0.7\text{--}1.0 \times 1.1\text{--}2.2 \mu\text{m}$. Cell growth appears to be monopolar since one cell end is usually more narrow and shorter, which indicates a budding process (Hirsch, 1974a). In PHBA medium (Labrenz et al., 1998), motility can be observed. Small daughter cells show predominantly tumbling motion and only rarely directed movements, but flagella were not observed.

Bchl *a* is found in strains EL-78, EL-83, EL-171, and EL-172^T in cell suspensions grown in the dark (Labrenz et al., 1999). Absorbances characteristic of bchl *a* with a large peak at 877–879 nm and smaller ones at 799–802 nm and 589–591 nm are similar to maxima found in bchl *a*-containing anoxygenic phototrophs (Biebl and Drews, 1969), but they differ from the maxima of bchl *a*-containing *Roseobacter denitrificans* and *Roseobacter litoralis* (Shiba, 1991a) or *Staleyia guttiformis* (Labrenz et al., 2000). Under identical growth conditions, bchl *a* could not be detected in suspensions or methanolic extracts of *R. tolerans* strains EL-52, EL-90, EL-164, or EL-222. Even concentrated extracts of these strains lacked bchl *a* when thin layer chromatography was applied. The *in vitro* absorption spectra of methanolic *R. tolerans* extracts show a large peak at 767–769 nm and smaller ones at 605–607, 699–700 nm, and around 350 nm. Unlike *Roseobacter denitrificans* or *Staleyia guttiformis*, the production of bchl *a* by these EL-strains is repressed in constant dim light.

Colony colors of strains EL-83 and EL-171 were white-beige to beige during the first six years of cultivation; at that time these

strains did not produce bchl *a*. Then the colony colors changed to light red and *in vivo* bchl *a* spectra had a small peak at 868–871 nm. Five months later, colonies of EL-83 became pinkish and since then this strain produces bchl *a* just as EL-78 and EL-172^T do. Methanolic extracts of all four bchl-producing EL-strains have identical absorption spectra. Unlike in *Roseobacter denitrificans*, vesicular intracytoplasmic membrane systems (Harashima et al., 1982) are not found in ultrathin sections of aerobically and dark grown cells of EL-172^T. *R. tolerans* has an absolute requirement for Na^+ , but not for K^+ , Mg^{2+} , Ca^{2+} , Cl^- , or SO_4^{2-} .

In the presence of available nitrogen, *Roseovarius tolerans* uses acetate, pyruvate, malate, succinate, butyrate, or glutamate, but not citrate, methanesulfonic acid, methanol, or α -D-glucose. Without any added nitrogen compounds, this species grows weakly on acetate, pyruvate, succinate, malate, or butyrate. Glutamate is used with and without an additional source of combined nitrogen. No growth on glucose anaerobically in the absence of nitrate. Metabolism of carbon sources tested with the Biolog system is described in Labrenz et al. (1999). Strain EL-222 grows microaerophilically.

The peptidoglycan of *R. tolerans* contains *m*-diaminopimelic acid. Respiratory quinone is Q10. Phospholipids present are: diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine as well as an unknown phospholipid and an aminolipid. The dominant fatty acid is $C_{18:1}$ (70.2%); other characteristic fatty acids are $C_{18:2}$ (10.6%), $C_{12:0} 2OH$ (2.4%), $C_{12:1} 3OH$ (3.6%), $C_{16:1}$ (0.8%), $C_{16:0}$ (6.2%), and $C_{18:0}$ (0.8%).

Comparative 16S rRNA gene sequence analysis shows *R. tolerans* to be a member of the *Alphaproteobacteria*. Highest 16S rRNA gene sequence relatedness (93–95%) is displayed with species of the genus *Ruegeria* (Uchino et al., 1998), viz. *R. algicola* (Lafay et al., 1995) as well as with *Roseobacter*, *Antarctobacter*, *Sagittula*, *Staleyia*, *Octadecabacter*, and *Sulfitobacter*. (See Fig. BXII.α.71 of the chapter describing the genus *Antarctobacter*.)

ENRICHMENT AND ISOLATION PROCEDURES

Two enrichment procedures may be used for *R. tolerans*: (1) Original water samples (0.5 ml) are spread directly on agar plates of medium PYGV prepared with water of 72‰ salinity or with 67‰ or 130‰ ASW salinity. The same procedure may be carried out with Sabouraud–Dextrose–Agar prepared with 72‰ salinity water. Incubation is at 4°C or 15°C in the dark or at $4.1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Pure cultures are isolated by several dilution transfers on the corresponding agar media. (2) Original water samples (50 ml) are amended with 1 ml of a filter-sterilized solution of 2.5 mg/ml Bacto Yeast Extract prepared in ASW (72‰); incubation at 15°C and in dim light of $4.1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

MAINTENANCE PROCEDURES

Strains of *Roseovarius tolerans* can initially be cultivated on PYGV or R2A agar with 25‰ or 40‰ ASW. After incubation at 8–33°C to allow abundant growth, cultures may be maintained at 4°C for 6 months. They can also be preserved indefinitely by lyophilization or frozen at –72°C in 15% glycerol (v/v).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

For the study of 16S rRNA gene fragments, PCR amplification is recommended as described by Hudson et al. (1993) and Labrenz et al. (1999).

DIFFERENTIATION OF THE GENUS *ROSEOVARIUS* FROM OTHER GENERA

Table BXII.α.73 lists characteristics of *Roseovarius* that differentiate it from other morphologically, physiologically, or chemotaxonomically similar organisms. Fig. BXII.α.71 of the chapter describing the genus *Antarctobacter* presents an unrooted tree showing phylogenetic relationships of *Roseovarius tolerans* with closely related *Alphaproteobacteria*.

TAXONOMIC COMMENTS

The combination of respiratory lipoquinone, fatty acid, and polar lipid data indicates that *R. tolerans* belongs with organisms (at the genus or family rank) within the *Alphaproteobacteria*. C_{18:1} is the characteristic fatty acid for the *Alphaproteobacteria* whereas C_{18:2} occurs in most cases in *R. tolerans* and *Roseobacter* spp. These

two groups are differentiated by fatty acids in lower proportions, such as C_{12:0 2OH} and C_{12:1 3OH} (*R. tolerans*), or C_{10:0 2OH} and C_{14:1 3OH} (*Roseobacter denitrificans* and *Roseobacter litoralis*) (Labrenz et al., 2000). When grown on Bacto Marine Broth (Difco), *Ruegeria algicola* lacks these characteristic fatty acids. However, the polar lipid patterns of *Roseovarius tolerans* and *Ruegeria algicola* are nearly identical (Labrenz et al., 1999) and they differ from those of *Roseobacter denitrificans*, *Roseobacter litoralis*, or *Antarctobacter heliothermus*. In addition, the mol% G + C ratio of *Ruegeria algicola* (64–65) resembles that of *R. tolerans* (62–64) but differs from that of other *Roseobacter* species (56.3–60.1). Comparative 16S rRNA gene sequence analysis, chemotaxonomic, biochemical, and physiological studies clearly show the close relationship of *Roseobacter denitrificans* with *Roseobacter litoralis*. On the other hand, *Ruegeria algicola* appears to be more related to, albeit different from, *Roseovarius tolerans*, as shown by physiological and fatty acid data (Labrenz et al., 1999).

16S rRNA gene sequencing data indicate the affiliation of *R. tolerans* with the *Alphaproteobacteria*; specifically, there is an association with the *Roseobacter* cluster, which also includes *Ruegeria*, *Staleyia*, *Sulfitobacter*, *Antarctobacter*, *Sagittula*, *Octadecabacter*, *Silicibacter*, and a *Prionitis lanceolata* gall symbiont. Sequence divergence values of >5% (4.7% to *Ruegeria algicola*) show *R. tolerans* to be phylogenetically distinct from all currently recognized members of the *Proteobacteria*. Furthermore, bootstrap resampling shows *R. tolerans* does not possess a particularly significant phylogenetic affinity with any individual species within the above-mentioned *Roseobacter* cluster.

List of species of the genus *Roseovarius*

- Roseovarius tolerans*** Labrenz, Collins, Lawson, Tindall, Schumann and Hirsch 1999, 145^{VP}
to'le.rans. L. part. adj. *tolerans* enduring stress conditions.

Rods with a size of 0.74–0.83 × 1.34–1.94 μm. Further morphological descriptions as for the genus. Physiological and nutritional characteristics are presented in Table BXII.α.74. Optimal growth occurs between 8°C and 33.5°C with salt concentrations of 1.0–8.0% NaCl or with 10–130‰ ASW. Optimal pH for growth is 5.9 to >9.0. Colonies on

medium PYGV or R2A + 25 (or 40‰) ASW are smooth, convex, and red, pinkish, beige to red, beige, or whitish beige. Isolated from hypersaline, meromictic, and heliothermal Ekho Lake, East Antarctica.

The mol% G + C of the DNA is: 62–64 (HPLC).

Type strain: EL-172, DSM 11457.

GenBank accession number (16S rRNA): Y11551.

Additional Remarks: Reference strains include EL-222, DSM 11463.

TABLE BXII.α.73. Differential characteristics of the genus *Roseovarius* and other morphologically, physiologically, or chemotaxonomically similar organisms^a

Characteristic	<i>Roseovarius tolerans</i>	<i>Antarctobacter heliothermus</i>	<i>Octadecabacter arcticus</i>	<i>Roseobacter litoralis</i>	<i>Ruegeria algicola</i>	<i>Sagittula stellata</i>	<i>Staleyia guttiformis</i>	<i>Sulfitobacter pontiacus</i>
Rosettes formed	–	+	–	–	–	+	+	+
Bud formation	+	+	–	–	–	nd	+	–
Bacteriochlorophyll <i>a</i>	d	–	–	+	–	–	v	–
Motility	d	d	–	+	+	+	+	+
Maximum length 10.9–33.6 μm	–	+	–	–	–	–	–	–
Growth at <8.5°C	+	+	+	+	–	+	+	+
Gelatin hydrolysis	–	+	–	+	+	–	–	–
Utilize methanol as a carbon source	–	–	–	–	–	+	–	–
Utilize citrate as a carbon source	–	+	d	+	+	+	–	–
Oxidase	W	+	–	+	+	+	+	+
Phosphatidylcholine	+	+	nd	–	+	nd	+	nd
Diphosphatidylglycerol	+	–	nd	+	+	nd	–	nd
Phosphatidylethanolamine	+	–	nd	–	+	nd	+	nd
C _{18:2} fatty acid	+	–	–	+	+	–	+	nd
C _{12:1 3OH} fatty acid	+	+	–	–	–	+	–	nd
C _{12:0 2OH} fatty acid	+	–	–	–	–	–	–	nd
Mol% G + C of DNA	62–64	62–63	57	56–58	64–65	65	55–56	62

^aFor symbols see standard definitions; nd, not determined; V, variable; W, weak reaction.

TABLE BXII.α.74. Other characteristics of *Roseovarius tolerans*^a

Characteristic	<i>R. tolerans</i>
Catalase activity	+
Oxidase activity	w
Bchl <i>a</i> absorption bands <i>in vivo</i> at 589–591 nm, 799–802 nm, and 877–879 nm	d
Bchl <i>a</i> absorption bands <i>in vitro</i> ^b at 605–607 nm, 699–700 nm, and 767–769 nm	d
Reduction of NO ₃ [–]	–
Requirement of vitamin B ₁₂	d
Requirement of thiamin or nicotinic acid	w
Requirement of pantothenate	–
Stimulation by biotin	+
Hydrolysis of gelatin, starch, or alginate	–

(continued)

TABLE BXII.α.74. (cont.)

Characteristic	<i>R. tolerans</i>
Hydrolysis of Tween 80 or DNA	d
Indole test	–
H ₂ S production	–
Voges–Proskauer test	–
Acid from glucose	–
Growth with succinate, glutamate, butyrate acetate, pyruvate, malate	+
Growth with citrate, α-D-glucose methanesulfonic acid	–
Susceptible to chloramphenicol and streptomycin	+
Susceptible to polymyxin B	–
Susceptible to penicillin G or tetracycline	d

^aFor symbols see standard definitions; w, weak reaction.^bIn methanolic extracts.Genus XX. *Rubrimonas* Suzuki, Muroga, Takahama, Shiba and Nishimura 1999b, 204^{VP}

CHRISTOPHER RATHGEBER AND VLADIMIR V. YURKOV

Ru.bri.mo' nas. L. adj. *ruber* reddish; Gr. n. *monas* unit; M.L. fem. n. *Rubrimonas* reddish monad.

***Rubrimonas* cells are Gram-negative, short to ovoid rods, 1.0–1.5 × 1.2–2.0 μm. Motile by polar flagella. Division occurs by binary fission. Forms pink circular colonies when grown on agar media. Pink coloration is due to the presence of carotenoid pigments. Cells grow heterotrophically under aerobic conditions and produce bacteriochlorophyll (Bchl) *a*. No growth occurs under anaerobic conditions even in the light.** Habitat is saline lakes.

The mol% G + C of the DNA is: 70.4–74.8.

Type species: *Rubrimonas cliftonensis* Suzuki, Muroga, Takahama, Shiba and Nishimura 1999b, 204.

FURTHER DESCRIPTIVE INFORMATION

Rubrimonas cliftonensis is the only species presently described. Phylogenetically, *Rubrimonas* falls within the *Alphaproteobacteria* forming a separate branch. The closest relative is *Rhodobacter veldkampii* with a 16S rDNA sequence similarity of 89.8%.

R. cliftonensis forms circular, smooth, slightly convex, opaque, pink colonies when grown on agar medium. Cells grown in PPES-II media (Suzuki et al., 1999b) are ovoid rods 1.0–1.5 × 1.2–2.0 μm. Optimal growth occurs at pH 7.5–8.0 and temperature 27–30°C. NaCl is required, with growth occurring in media supplemented with 0.5–7.5% NaCl.

R. cliftonensis is an obligately aerobic heterotroph that produces Bchl *a* under aerobic conditions. Incapable of anaerobic growth even under illumination. A wide variety of organic substrates can be used to support heterotrophic growth, which is typical of other aerobic phototrophic bacteria. Cells utilize D-glucose, L-arabinose, D-fructose, D-galactose, D-ribose, D-xylose, citrate, glycolate, pyruvate, and ethanol. Acids are produced from sugars. Urease, phosphatase, and nitrate reductase are produced (See genus Table BXII.α.71 in Genus *Roseivivax*).

The absorption spectrum of membrane fractions shows a major absorbance peak at 806 and a smaller peak at 871 nm, indicating the presence of Bchl *a* organized into light-harvesting II and light-harvesting I complexes, respectively. This absorption

spectrum is similar to that found among members of the genus *Roseobacter*, indicating a similar organization of the photosynthetic apparatus (Nishimura et al., 1994).

The major cellular fatty acid is C_{18:1}, making up 68–70% of the cellular fatty acid profile. C_{16:0}, C_{18:0}, C_{20:0}, C_{19:1}, and C_{14:0} 3OH are found in smaller quantities. The principal ubiquinone is Q-10, making up 98–99% of the quinone content; trace amounts of the quinones Q-8 and Q-9 are also present.

ENRICHMENT AND ISOLATION PROCEDURES

R. cliftonensis was isolated from water samples taken from Lake Clifton, an isolated saline lake on the west coast of Australia. Lake Clifton is a ground water-fed lake, which exhibits a large range in salinity from 14.5 kg/m³ to 31.5 kg/m³ (~1.5–3.2%) (Rosen et al., 1996). Samples were plated on PPES-II agar medium (Suzuki et al., 1999b). Pure cultures were obtained by replating of separate colonies.

MAINTENANCE PROCEDURES

R. cliftonensis can be maintained on PPES-II slant agar culture medium. Long-term storage procedures have not been described.

DIFFERENTIATION OF THE GENUS *RUBRIMONAS* FROM OTHER GENERA

Genus *Rubrimonas* can be differentiated from other genera based on 16S rRNA gene sequence, as well as a relatively high mol% G + C content of 74.8. *R. cliftonensis* forms a distinct branch only distantly related to other phototrophic and nonphototrophic genera. The nearest relative is *Rhodobacter veldkampii*, a purple nonsulfur bacterium. *Rubrimonas* is an obligately aerobic phototrophic bacterium, which forms circular pink colonies on agar media due to the presence of carotenoid pigments. It can be distinguished from nonphototrophic genera by absorption spectrum peaks at 806 and 871 nm, corresponding to the presence

of Bchl *a*. Aerobic phototrophic bacteria are distinguished from closely related purple nonsulfur bacteria by their inability to utilize light for anaerobic photosynthetic growth.

TAXONOMIC COMMENTS

Shiba et al. (1991) described several strains of aerobic bacteriochlorophyll *a*-containing bacteria from saline locations on the east and west coasts of Australia. Thirty-seven strains were divided into four groups (GI, GII, GIII, and GIV) based on cell color, type of absorption spectrum, and cell morphology. DNA–DNA hybridization studies have shown that the two strains making up the GIII group were not closely related to members of the other

three groups (Nishimura et al. 1994), and they were subsequently described as *Rubrimonas cliftonensis*.

16S rDNA sequence analysis shows that *Rubrimonas* forms a separate branch within the *Alphaproteobacteria*, with no close relatives. The nearest relative is *Rhodobacter veldkampii* with 89.8% sequence similarity.

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- Yurkov, V.V. and J.T. Beatty. 1998. Anoxygenic aerobic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

List of species of the genus *Rubrimonas*

1. ***Rubrimonas cliftonensis*** Suzuki, Muroga, Takahama, Shiba and Nishimura 1999b, 204^{VP}
clif.to.nen'sis. M.L. adj. *cliftonensis* referring to Lake Clifton, Australia, the source of the type strain.

Gram-negative short to ovoid rods (1.0–1.5 × 1.2–2.0 μm), motile possessing polar flagella, divide by means of binary fission. Form circular, pink colonies when grown on agar media. Cells produce Bchl *a* under aerobic conditions, giving rise to absorption spectrum peaks at 806 and 871 nm, corresponding to Bchl *a* incorporated into light-harvesting II and light-harvesting I complexes respectively. Produce carotenoid pigments. Obligately aerobic heterotroph, incapable of anaerobic growth even under illuminated conditions. Utilize D-glucose, L-arabinose, D-fructose, D-galactose, D-ribose, D-xylose, citrate glycolate, pyruvate,

and ethanol. Do not utilize acetate, fumarate, DL-lactate, DL-malate, succinate, methanol, L-glutamate, lactose, or sucrose. Acids are produced from sugars. Do not hydrolyze starch, gelatin, alginate, or Tween 80. Cells produce catalase, oxidase, nitrate reductase, phosphatase, and urease. Voges–Proskauer test and ONPG reaction are negative. Produce indole but not H₂S.

NaCl is required, with growth occurring at concentrations ranging from 0.5 to 7.5%. Optimal growth occurs at pH 7.5–8.0 and temperature 27–30°C. Cells are resistant to penicillin and are susceptible to chloramphenicol, streptomycin, and tetracycline. Habitat: saline lake water from Lake Clifton, Australia.

The mol% G + C of the DNA is: 74.0–74.8 (HPLC).

Type strain: Och317, CIP 105913, JCM 10189.

GenBank accession number (16S rRNA): D85834.

Genus XXI. ***Ruegeria*** Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208)

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Rue.ger'ia. M.L. -ia ending; M.L. fem. n. *Ruegeria* honoring Rueger, a German microbiologist, for his contribution to the taxonomy of marine species of *Agrobacterium*.

Gram-negative, **ovoid to rod-shaped cells**, 0.6–1.6 × 1.0–4.0 μm. Motile by polar flagella, or nonmotile. Do not form spores. **Aerobic**. Oxidase and catalase positive. No photosynthetic growth. Bacteriochlorophyll *a* is absent. **Major quinone is ubiquinone 10**.

The mol% G + C of the DNA is: 55–59.

Type species: Ruegeria atlantica (Rüger and Höfle 1992) Uchino, Hirata, Yokota and Sugiyama 1999, 1 (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium atlanticum* Rüger and Höfle 1992, 141.)

List of species of the genus *Ruegeria*

1. ***Ruegeria atlantica*** (Rüger and Höfle 1992) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium atlanticum* Rüger and Höfle 1992, 141.)
at.lan'ti.ca. M.L. adj. *atlantica* pertaining to the Atlantic Ocean as the locality.

Gram-negative, ovoid to rod-shaped cells, 0.6–1.6 × 1.0–4.0 μm. Motile by polar flagella or nonmotile. Do not form spores. Aerobic. Oxidase and catalase positive. Non-photosynthetic. Bacteriochlorophyll *a* is absent. Nonmotile. Nitrate reduced to nitrite. Seawater or Na⁺ required for growth. Major fatty acid is C_{18:1}. 3-Hydroxy fatty acids are C_{12:0} 3OH and C_{14:1} 3OH. 2-Hydroxy fatty acid is C_{16:0} 2OH. Major quinone is ubiquinone 10.

The mol% G + C of the DNA is: 55–58.

Type strain: 1480, ATCC 700000, CIP 105975, DSM 5823, IAM 14463, IFO 15792.

GenBank accession number (16S rRNA): D88526.

2. ***Ruegeria algicola*** (Lafay, Ruimy, Rausch de Traubenberg, Breittmayer, Gauthier and Christen 1995) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 209) (*Roseobacter algicola* Lafay, Ruimy, Rausch de Traubenberg, Breittmayer, Gauthier and Christen 1995, 295.)
al.gi'co.la. L. n. *alga* algae; L. subst. *cola* dweller; M.L. n. *algicola* algae dweller.

Gram-negative, ovoid to rod-shaped cells, 0.6–1.6 × 1.0–4.0 μm. Motile by means of one or two subpolar flagella. Do not form spores. Aerobic. Oxidase and catalase positive.

Not photosynthetic. Bacteriochlorophyll *a* is absent. Cells are ovoid during exponential growth. Colonies on salt-containing agar are beige when young, pinkish beige after 96 h. Optimal temperature 25–30°C. No denitrification. The following tests are positive: oxidase, catalase, gelatinase, esculinase, β -galactosidase, and amylase. Do not accumulate polyhydroxybutyrate. Isolated from a culture of the toxin-producing dinoflagellate *Prorocentrum lima* PLV2. Require Na^+ for growth. Major fatty acid is $\text{C}_{18:1}$. 3-Hydroxy fatty acids are $\text{C}_{12:0} \text{ 3OH}$, $\text{C}_{10:0} \text{ 3OH}$, and $\text{C}_{14:1} \text{ 3OH}$. 2-Hydroxy fatty acids are absent. Major quinone is ubiquinone 10.

The mol% G + C of the DNA is: unknown.

Type strain: FF3, ATCC 51440, DSM 10251, IAM 14591.

GenBank accession number (16S rRNA): X78315.

3. *Ruegeria gelatinovorans* (Rüger and Höfle 1992) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publica-

tion: Uchino, Hirata, Yokota and Sugiyama 1998, 209) (*Agrobacterium gelatinovorum* Rüger and Höfle 1992, 141.) *ge.la.ti.no'vor.ans*. M.L. gelatin; L. v. *vor*o to devour; M.L. adj. *gelatinovorans* gelatin-devouring.

Gram-negative, ovoid to rod-shaped cells, $0.6\text{--}1.6 \times 1.0\text{--}4.0 \mu\text{m}$. Motile by means of polar flagella. Do not form spores. Aerobic. Oxidase and catalase positive. Not photosynthetic. Bacteriochlorophyll *a* is absent. Nitrate reduced to nitrite. Acids produced from glycerol but not glucose, fructose, maltose, or xylose. Isolated from seawater of the Baltic Sea. Require seawater or Na^+ for growth. Major fatty acids are $\text{C}_{18:1}$ and $\text{C}_{18:0}$. 3-Hydroxy fatty acid is $\text{C}_{12:0} \text{ 3OH}$. 2-Hydroxy fatty acids are absent. Major quinone is ubiquinone 10.

The mol% G + C of the DNA is: 59.

Type strain: B6, ATCC 25655, DSM 5887, IAM 12617.

GenBank accession number (16S rRNA): D88523.

Genus XXII. *Sagittula* González, Mayer, Moran, Hodson and Whitman 1997b, 778^{VP}

JOSÉ M. GONZÁLEZ

Sa.git' tu.la. L. fem. n. *sagittula* small arrow, referring to the shape of the bacterium.

Rod-shaped cells, $0.9 \times 2.3 \mu\text{m}$. Gram negative. Cells attach by one pole to particles of cellulose and lignocellulose by means of a polar holdfast structure that can be seen by electron microscopy. Endospores and cysts are not formed. Capsules are produced. **Intracellular granules of polyhydroxybutyrate are formed.** **Aerobic, having a strictly aerobic type of metabolism with oxygen as the terminal electron acceptor.** Do not denitrify. Colonies are nonpigmented. Bacteriochlorophyll *a* is not present. **Oxidase and catalase positive.** **Sea-salt-based medium is required for growth.** Chemoorganotrophic. Methanol, various carbohydrates and amino acids, and some aromatic compounds such as *p*-coumarate, cinnamate, ferulate, and vanillate are utilized. Organic nitrogen compounds, ammonium, and nitrate serve as nitrogen sources. **Cellulose is hydrolyzed. Synthetic lignin preparations are partially solubilized and mineralized in the presence of glucose.** 16S rRNA gene sequence analysis positions this genus in a group of marine bacteria (except for the moderate halophile *Silicibacter*) within the family *Rhodobacteraceae*. Isolated from a salt marsh at 2% salinity on the coast of Georgia, USA, by enrichment with lignin-rich pulp mill effluent.

The mol% G + C of the DNA is: 65.0.

Type species: *Sagittula stellata* González, Mayer, Moran, Hodson and Whitman 1997b, 778.

FURTHER DESCRIPTIVE INFORMATION

As seen by electron microscopy, each cell has a holdfast structure at one pole and the cell envelope has numerous surface vesicles derived from the outer membrane (Fig. BXII.α.80). Cells form rosettes and aggregates, especially at the stationary phase of growth.

Sagittula contains the following major fatty acids: $\text{C}_{16:0}$, $\text{C}_{18:0}$, $\text{C}_{12:1} \text{ 3OH}$, $\text{C}_{19:0} \text{ cyclo } \omega 8$, $\text{C}_{18:1} \omega 7$, $\text{C}_{18:1} \omega 9$, and $\text{C}_{18:1} \omega 12$.

The only species in the genus was isolated from a salt marsh at 2% salinity on the coast of Georgia, USA, by enrichment with the high-molecular-weight fraction of pulp mill effluent. Although the species so far includes only one strain, closely related organisms were detected by molecular techniques in unpolluted

coastal water, which receives high inputs of lignocellulosic material from aquatic plants.

ENRICHMENT AND ISOLATION PROCEDURES

The type strain was isolated on YTSS¹ agar plates from a marine enrichment community growing on the high-molecular-weight fraction of a black liquor sample from pulp mill effluent, which is rich in lignin, lignin byproducts, and other plant polymers in a smaller proportion (González et al., 1997b). The original inoculum was from a salt marsh on the coast of Georgia, USA. The enrichment medium consisted of filter-sterilized seawater containing 5 mM NH_4NO_3 , 1 mM KH_2PO_4 , and the liquor fraction at a concentration of 20 mg C per liter. This medium was inoculated with cloudy, brown-green seawater from the salt marsh. Flasks were incubated aerobically with shaking. After enrichment, the organism was isolated on YTSS agar plates incubated at room temperature.

MAINTENANCE PROCEDURES

The type strain is maintained in YTSS broth with 15% glycerol and 15% DMSO at -70°C . Lyophilized cultures are also used.

DIFFERENTIATION OF THE GENUS *SAGITTULA* FROM OTHER GENERA

Table BXII.α.75 lists characteristics differentiating *Sagittula* from other aerobic marine bacteria. *Sagittula* does not oxidize sulfite, and this characteristic distinguishes it from *Sulfitobacter*. The absence of bacteriochlorophyll *a* production, major fatty acids, and the mol% G + C content of the DNA distinguish it from *Roseobacter denitrificans*, *Roseobacter litoralis*, and *Octadecabacter*. Temperature tolerance is different from *Octadecabacter* and *Silicibacter* (Table BXII.α.75). 16S rRNA gene sequences of *Sagittula* and "*Marinosulfonomonas*" show a low level of homology (90%).

1. YTSS agar (g/l distilled water): yeast extract, 4.0; tryptone (Difco), 2.5; sea salts (Sigma Chemical Co., St. Louis, MO), 20; and agar, 18. YTSS broth is the same except that agar is omitted.

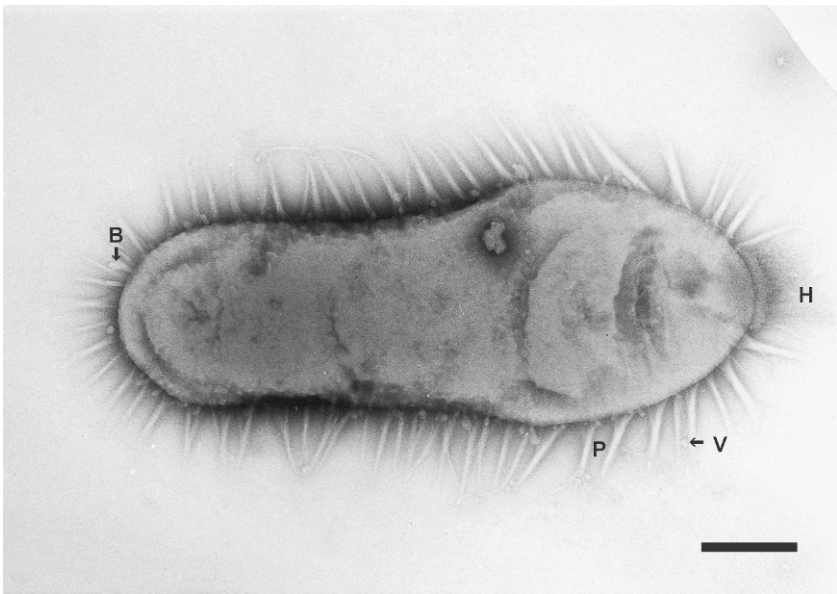


FIGURE BXII.α.80. Electron micrograph of *Sagittula stellata*. Negatively stained. Cells are covered by polysaccharides fibrils (*P*) and blebs (*B*). Vesicles (*V*) are also seen unattached. Holdfast structure (*H*) at the wider cell pole is indicated. The cells attach to cellulose and lignocellulose particles by the wider pole. Bar = 0.2 μm. (Courtesy of F. Mayer).

TABLE BXII.α.75. Differential characteristics of *Sagittula* and some phylogenetically closely related bacteria^a

Characteristic	<i>Sagittula stellata</i>	<i>“Marinosulfonomonas”</i>	<i>Octadecabacter</i> species	<i>Roseobacter denitrificans</i>	<i>Ruegeria algicola</i>	<i>Silicibacter</i>	<i>Sulfitobacter pontiacus</i>
Cells arranged in rosettes	+	+	–	–	–	–	+
Gas vacuoles	–	–	+	–	–	+	–
Oxidase	+	+	–	+	+	+	+
Nitrate red	–	–	–	+ / –	–	+	–
Bchl <i>a</i>	–	–	–	+	–	–	–
Growth temperature, °C	10–41	20–37	4–15	2–30	10–37	22–50	4–35
Optimal temperature, °C	30	30			25–30	45	22–25
Utilization of:							
DMSP	+	–					
Formate	+	+					
Methanol	+	+	–	–	–		–
Sulfite oxidation	–			–			+
Mol% G + C DNA	65		56–57	56–60	55–59	66	58–62

^aFor symbols see standard definitions.

TAXONOMIC COMMENTS

Based on 16S rRNA gene sequence analysis, the closest related genera are *Sulfitobacter*, *Roseobacter*, *Ruegeria*, *Octadecabacter*, *Silici-*

bacter, and *“Marinosulfonomonas”*. The genus has a signature secondary structure since a deletion corresponding to Helix 11 of the 16S rRNA occurs. This deletion spans 11 bases starting at position 200 (*E. coli* numbering system).

List of species of the genus *Sagittula*

1. ***Sagittula stellata*** González, Mayer, Moran, Hodson and Whitman 1997b, 778^{VP}
stel.la'ta. L. adj. *stellata* starry, here referring to the cell arrangement.
Colonies on marine agar 2216 are light cream colored. The characteristics are as described for the genus with the following additional information. The cells show polarity, the width of one half is greater than that of the other. The holdfast structure is located at the thicker pole. Vesicles are not only seen on the cell surface but also are free in

the medium. The cells occur singly and arranged as rosettes, especially at stationary phase, and form short chains in marine broth 2216.
Growth occurs between pH 5.5 and 8.5; optimal, 7.5. The temperature range for growth is 4–41°C; optimal, 30°C. Sea-salt-based medium is required for growth. Vitamins are required for optimal growth. Does not grow in the absence of a source of fixed nitrogen.
Growth occurs on methanol, the salts of various organic acids, monosaccharides, disaccharides, amino acids, and ar-

omatic compounds. Among the latter are lignin-related compounds such as *p*-coumarate, cinnamate, ferulate, and vanillate. Cells also utilize organic sulfur compounds and similar compounds that are produced by aquatic plants and algae, such as dimethylsulfoniopropionate, 3-mercaptopropionate, methanethiol, and glycine betaine. Strain E-37 quickly oxidizes dimethylsulfide to dimethylsulfoxide in aer-

obic conditions without further degradation of dimethylsulfoxide (González et al., 1999). Strain E-37 was able to solubilize and partially mineralize artificial lignin in the presence of glucose.

The mol% G + C of the DNA is: 65.0 (HPLC).

Type strain: E-37, ATCC 700073; CIP 105237, DSM 11524.

GenBank accession number (16S rRNA): U58356.

Genus XXIII. *Staley* Labrenz, Tindall, Lawson, Collins, Schumann and Hirsch 2000, 310^{VP}

MATTHIAS LABRENZ AND PETER HIRSCH

Staley.a. M.L. fem. n. *Staley* named after the American microbiologist J.T. Staley in recognition of his work on budding and appendaged bacteria and his contributions to polar microbiology.

Rods, one or both cell poles narrower, multiplying by monopolar growth, i.e., by a budding process. Gram negative. Cells may be motile. No resting stages known. **Aerobic**, with a strictly respiratory type of metabolism. **Intracellular granules of poly- β -hydroxybutyrate (PHB) are present**. Cells have a weak requirement for Na⁺. Temperature range for growth <4–32°C, salinity range <10 to <150‰ of artificial seawater (ASW), NaCl tolerance range <1.0–4.0% and pH tolerance range 5.3–6.5 to >9. Oxidase and weakly catalase positive. Do not grow photoautotrophically with H₂/CO₂ (80:20) or photoorganotrophically with acetate or glutamate. Bacteriochlorophyll *a* (BChl *a*) may be produced. Occur in marine habitats.

The mol% G + C of the DNA is: 55–56.

Type species: *Staley* *guttiformis* Labrenz, Tindall, Lawson, Collins, Schumann and Hirsch 2000, 310.

FURTHER DESCRIPTIVE INFORMATION

Visible growth of *S. guttiformis* appears at 20°C after 3–5 d on medium PYGV (Staley, 1968) prepared with 25‰ artificial seawater (ASW; Lyman and Fleming, 1940). Colonies are circular, smooth, convex, 1–3 mm in diameter and beige, older colonies also pink. Cell sizes: 1.0–1.5 × 1.5–8.9 µm. Cell growth appears to be monopolar since one cell end is usually more narrow and shorter, which indicates a budding process (Hirsch, 1974a). Rosettes are formed frequently. Cells are motile with one flagellum.

Bacteriochlorophyll *a* is found in older, dark-grown cell suspensions of *Staley* *guttiformis*. Absorbance values characteristic of BChl *a* have a large peak at 861–862 nm, and smaller ones at 800–802 nm and 590–592 nm. They differ from maxima of the BChl *a*-containing *Roseobacter denitrificans* and *Roseobacter litoralis* (Shiba, 1991a) or *Roseovarius tolerans* (Labrenz et al., 1999). Unlike *Roseovarius tolerans*, the production of BChl *a* by *S. guttiformis* is not totally repressed in constant dim light.

S. guttiformis has a weak requirement for Na⁺, but not for K⁺, Mg²⁺, Ca²⁺, Cl[−] or SO₄^{2−}.

Cells do not grow on glucose anaerobically in the absence of nitrate. Metabolism of carbon sources tested with the Biolog system is described by Labrenz et al. (2000). Acetoin or acids are not produced from glucose. Neither sulfide nor indole is produced. Do not reduce nitrate anaerobically.

The peptidoglycan of *S. guttiformis* contains *m*-diaminopimelic acid. Respiratory quinone is Q-10. Phospholipids present are: phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine as well as an unknown aminolipid. The predominant

fatty acid is C_{18:1 ω7c} (79.7%); other characteristic fatty acids are C_{10:0 3OH} (5.9%), C_{14:1 3OH} (2.1%), C_{16:0} (3.9%), C_{18:0} (0.7%), C_{18:2} (5.3%), and C_{19:1} (1.4%).

Comparative 16S rRNA gene sequence analysis shows that *S. guttiformis* is a member of the *Alphaproteobacteria*. Highest 16S rRNA gene sequence relatedness (98%) is displayed with *Sulfitobacter mediterraneus* (Pukall et al., 1999), a new species described simultaneously with *Staley*. In addition, high sequence relatedness is observed with *Sulfitobacter pontiacus* (Sorokin, 1995), *S. brevis* (Labrenz et al., 2000), *Roseobacter denitrificans* and *Roseobacter litoralis* (Shiba, 1991a). Other species belonging to the *Alphaproteobacteria* show lower levels of relatedness. An unrooted tree depicting the phylogenetic relationships of *S. guttiformis* and its closest relatives is shown in Fig. BXII.α.71 of the chapter describing the genus *Antarctobacter*. The results of treeing analyses showed that this species phylogenetically clusters with *Sulfitobacter* spp.

ENRICHMENT AND ISOLATION PROCEDURES

An enrichment procedure used for *S. guttiformis*: Original water samples (1.0 ml) are spread directly on medium PYGV agar plates prepared with water of 15‰ salinity. Incubation is at 15°C at 4.1 µmol photons m^{−2} s^{−1}.

MAINTENANCE PROCEDURES

Staley *guttiformis* can initially be cultivated on PYGV agar with 10–40‰ ASW. After incubation at 12–20°C to allow abundant growth, cultures may be maintained in a refrigerator (4°C) for 6 months. They can also be preserved by lyophilization or frozen at −72°C in 15% glycerol (v/v).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

For the study of 16S rRNA gene fragments, PCR amplification is recommended as described by Hudson et al. (1993) and Labrenz et al. (1998).

DIFFERENTIATION OF THE GENUS *STALEYA* FROM OTHER GENERA

Table BXII.α.76 lists characteristics of *Staley* that differentiate it from other morphologically, physiologically, or chemotaxonomically similar organisms. Fig. BXII.α.71 of the chapter describing the genus *Antarctobacter* presents an unrooted tree showing phylogenetic relationships of *Staley* *guttiformis* with closely related *Alphaproteobacteria*.

TAXONOMIC COMMENTS

Sequencing of 16S rRNA genes confirmed *S. guttiformis* is a member of the *Alphaproteobacteria*. The combination of respiratory lipoquinone, fatty acid, and polar lipid data also indicates that it belongs with bacteria (above the species rank) of the *Alphaproteobacteria* (Labrenz et al., 2000). Comparative 16S rRNA gene sequence analysis shows that *S. guttiformis* is specifically associated with the *Sulfitobacter*–*Roseobacter* cluster, but not with *Ruegeria algicola* or *Roseobacter gallaeciensis*. It is evident from treeing analyses that the genus *Roseobacter*, as currently recognized, is interspersed with several other taxa. This is also reflected by the affiliation of the former *Roseobacter algicola* with the genus *Ruegeria* (Uchino et al., 1998).

The chemotaxonomic data indicate that members of the genus *Roseobacter* (i.e., *R. litoralis* and *R. denitrificans*) have a distinctive polar lipid composition, in which both species have phosphatidylglycerol, diphosphatidylglycerol, and an unidentified amino lipid as the major components. In contrast, *S. guttiformis*, *Sulfitobacter brevis*, and *S. pontiacus* all synthesized phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine, together with the same unidentified amino lipid. This polar lipid composition serves to distinguish these two groups from one another.

Phylogenetically, *S. guttiformis* is closely related to *Sulfitobacter* spp. Morphologically, it is also more similar to *Sulfitobacter* than to *Roseobacter* strains: its cells multiply by monopolar growth (a

budding process) and form rosettes. The ability to oxidize sulfite and thereby to increase growth characterizes *S. pontiacus* (Sorokin, 1995). *S. mediterraneus* grows also to higher cell densities with 10 mM sulfite in an acetate-supplemented medium (Pukall et al., 1999). *S. brevis* tolerates lower initial sulfite concentrations in an acetate-supplemented medium than *S. pontiacus*, but not so low as *Staleyia guttiformis* (Labrenz et al., 2000).

Especially, chemotaxonomic data indicate differences between *Staleyia* and *Sulfitobacter*. *Staleyia* does not synthesize the fatty acids C_{16:1}, C_{17:1}, C_{17:0}, or C_{18:1 10CH₃} as well as the phospholipid diphosphatidylglycerol. Examination of the mol% G + C base content showed that the value for *Staleyia* was about 6% lower than that for the *Sulfitobacter* type species, *S. pontiacus*.

Other differences concerned BChl *a*, which was present in *Staleyia guttiformis*, as shown by *in vivo* absorption bands at 800–802 and 861–862 nm. These peaks were clearly absent from *Roseobacter* spp. and *Sulfitobacter* cells. The importance of BChl *a* for a taxonomic separation of these aerobic bacteria has been questioned (Labrenz et al., 1999).

In conclusion, bacteria of the *Staleyia*–*Sulfitobacter*–*Roseobacter* cluster appear to be phylogenetically quite closely related, but are phenotypically very diverse, which makes their taxonomic separation difficult. 16S rDNA sequence data and similarities in chemotaxonomic data make it appear likely that the genera *Staleyia*, *Sulfitobacter*, and *Roseobacter* share a common ancestor (Labrenz et al., 1999). Presently, *Staleyia* is placed between *Roseobacter* (Shiba, 1991a) and *Sulfitobacter* (Sorokin, 1995).

TABLE BXII.α.76. Differential characteristics of the genus *Staleyia* and other morphologically, physiologically, or chemotaxonomically similar organisms^a

Characteristic	<i>Staleyia</i>	<i>Antarctobacter heliothermus</i>	<i>Octadecabacter arcticus</i>	<i>Roseobacter litoralis</i>	<i>Roseovarius tolerans</i>	<i>Ruegeria algicola</i>	<i>Sagittula stellata</i>	<i>Sulfitobacter mediterraneus</i>	<i>Sulfitobacter pontiacus</i>
Rosettes formed	+	+	–	–	–	–	+	+	+
Budding cell division	+	+	–	–	+	–	nd	+	+
Oxidase	+	+	–	+	+	+	+	+	+
BChl <i>a</i>	v	–	–	+	+	–	–	–	–
Growth at 4°C	+	+	+	+	+	–	+	+	+
Growth at 37°C	–	+	–	–	+	+	+	–	–
Tween 80 hydrolysis	+	–	nd	+	–	–	–	nd	–
Gelatin hydrolysis	–	+	–	+	–	+	–	+	–
Citrate utilized as carbon source	–	+	v	+	–	+	+	+	–
Butyrate utilized as carbon source	–	+	–	–	w	–	+	+	+
<i>Fatty acids:</i>									
C _{10:0} 3OH	+	–	+	+	–	–	–	+	+
C _{12:1} 3OH	–	+	–	–	+	–	+	–	–
C _{12:0} 2OH	–	–	–	–	+	–	–	–	–
C _{14:1} 3OH	+	–	–	+	–	–	–	–	+
C _{16:1}	–	+	+	–	+	–	–	+	+
C _{17:1}	–	–	–	–	–	–	–	+	+
C _{17:0}	–	–	–	–	–	–	–	+	+
C _{18:2}	+	–	–	+	+	+	–	–	–
C _{18:1 ω9c}	–	–	–	–	–	–	–	+	–
C _{18:1 10CH₃}	–	–	–	–	–	–	–	+	+
C _{18:1 18CH₃}	–	–	–	–	–	–	–	+	–
C _{19:0} cyclo	–	+	–	–	–	–	+	–	–
C _{19:1}	+	–	–	–	–	–	–	–	–
Diphosphatidylglycerol	–	–	nd	+	+	+	nd	nd	+
Phosphatidylethanolamine	+	–	nd	–	+	+	nd	nd	+
Phosphatidylcholine	+	+	nd	–	+	+	nd	nd	+
Mol% G + C content	55–56	62–64	57	56–59	63	64–65	65	59	62–63

^aFor symbols see standard definitions; nd, not determined; w, weak reaction.

List of species of the genus *Staleyia*

1. ***Staleyia guttiformis*** Labrenz, Tindall, Lawson, Collins, Schumann and Hirsch 2000, 310^{VP}
gut.ti.for' mis. L. fem. n. *gutta* the drop; M.L. *guttiformis* drop shaped.

Rods with average size of $1.1 \times 1.8 \mu\text{m}$. Further morphological description as for the genus. Physiological and nutritional characteristics are presented in Table BXII.α.77. Optimal growth occurs between 12 and 20°C with 1.0% NaCl or 10–40‰ ASW. Optimal pH for growth is 7.0–8.5. Colonies on medium PYGV are smooth, convex, and beige, later pink. Isolated from hypersaline, meromictic, and heliothermal Ekho Lake, East Antarctica.

The mol% G + C of the DNA is: 55–56 (HPLC).

Type strain: EL-38, DSM 11458.

GenBank accession number (16S rRNA): Y16427.

TABLE BXII.α.77. Other characteristics of *Staleyia guttiformis*^a

Characteristic	<i>S. guttiformis</i>
Catalase activity	w
Bchl <i>a</i> absorption bands <i>in vivo</i> at 590–592 nm, 800–802 nm and 861–862 nm	v
Aerobic reduction of NO ₃ [−] to NO ₂ [−]	+
Requirement of vitamin B ₁₂	−
Requirement of thiamin, biotin, or nicotinic acid	w
Requirement of pantothenate	+
Hydrolysis of starch or alginate	−
Hydrolysis of DNA	+
Growth with succinate, glutamate, acetate, pyruvate, malate	+
Growth with α-D-glucose	w
Growth with methanesulfonic acid or methanol	−
Susceptible to chloramphenicol, penicillin G, tetracycline, polymyxin B, nalidixic acid or streptomycin	+

^aFor symbols see standard definitions; w, weak reaction.

Genus XXIV. *Stappia* Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208)

THE EDITORIAL BOARD

Stap.pi.a. M.L. dim. *-ia* ending; M.L. fem. n. *Stappia* honoring Stapp, a Belgian microbiologist, for his contribution to the taxonomy of marine species of *Agrobacterium*.

Gram-negative **rods**, 0.6–1.0 × 2.0–4.0 μm. Motile by means of polar flagella. Do not form spores. **Aerobic**. Nonphototrophic. Oxidase and catalase positive. Seawater or Na⁺ required for growth. Major fatty acids are C_{18:1} and C_{18:0}. 3-Hydroxy fatty acid is C_{14:0} 3OH. 2-Hydroxy fatty acids are absent. **Major quinone is ubiquinone 10**. Isolated from marine sediment and seawater.

The mol% G + C of the DNA is: 59.

Type species: ***Stappia stellulata*** (Rüger and Höfle 1992) Uchino, Hirata, Yokota and Sugiyama 1999, 1 (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium stellulatum* Rüger and Höfle 1992, 141.)

List of species of the genus *Stappia*

1. ***Stappia stellulata*** (Rüger and Höfle 1992) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208) (*Agrobacterium stellulatum* Rüger and Höfle 1992, 141.)
stel' lu.ta.ta. M.L. fem. n. *stella* star-shaped; M.L. adj. *stellulata* star-shaped.

Gram-negative rods, 0.6–1.0 × 2.0–4.0 μm. Motile by means of polar flagella. Do not form spores. Aerobic. Non-phototrophic. Oxidase and catalase positive. Seawater or Na⁺ required for growth. Nitrate is not reduced to nitrate but is reduced to gas. No acid from glucose, maltose, or mannitol after 4–6 weeks. Major fatty acids are C_{18:1} and C_{18:0}. 3-Hydroxy fatty acid is C_{14:0} 3OH. 2-Hydroxy fatty acids are absent. Major quinone is ubiquinone 10. Isolated from marine sediment and seawater.

The mol% G + C of the DNA is: 59.

Type strain: ATCC 15215, CIP 105977, DSM 5886, IAM 12621, IFO 15764.

GenBank accession number (16S rRNA): D88525.

2. ***Stappia aggregata*** (ex Ahrens 1968) Uchino, Hirata, Yokota and Sugiyama 1999, 1^{VP} (Effective publication: Uchino, Hirata, Yokota and Sugiyama 1998, 208.)
ag.gre.ga'ta. L. adj. *aggregatus* joined together, referring to the frequent formation of rosettes.

Gram-negative rods, 0.6–1.0 × 2.0–4.0 μm. Motile by means of polar flagella. Do not form spores. Aerobic. No photosynthetic growth. Oxidase and catalase positive. Seawater or Na⁺ required for growth. Nitrate is not reduced to nitrate but is reduced to gas. No acid from glucose or xylose. Weak or variable acid production from fructose, glycerol, and maltose after 4–6 weeks. Major fatty acids are C_{18:1} and C_{18:0}. 3-Hydroxy fatty acid is C_{14:0} 3OH. 2-Hydroxy fatty acids are absent. Major quinone is ubiquinone 10. Isolated from marine sediment and seawater.

The mol% G + C of the DNA is: 59.

Type strain: B1, ATCC 25650, IAM 12614, NCMB 2208.

GenBank accession number (16S rRNA): D88520.

Genus XXV. Sulfitobacter Sorokin 1996, 362^{VP} (Effective publication: Sorokin 1995, 304)

DIMITRY Y. SOROKIN, FRED A. RAINEY, RICHARD I. WEBB AND JOHN A. FUERST

Sul.fi.to.bac'ter. M.L. n. *sulfutum* sulfite; M.L. masc. form *bacter* Gr. neut. n. *bactrum* rod; M.L. masc. n. *Sulfitobacter* sulfite rod.

Rod-shaped cells 0.5–1.0 × 1.0–3.0 µm. Often dumbbell shaped, with a tendency for polar growth. **Motile by means of 1–5 subpolar flagella.** Gram negative. Inside the cells, the nucleoid and polar region are often in separate compartments. **Obligately aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Obligately chemoorganotrophic, but can use energy from the oxidation of thiosulfate and sulfite during growth in the presence of an organic carbon source. Cells cannot grow autotrophically with sulfur compounds or hydrogen as electron donors, but are able to oxidize thiosulfate, sulfur, and sulfite to sulfate.** Can grow in presence of at least 10 mM sulfite; some strains can grow with up to 60 mM sulfite. **NaCl is required for growth.** Mesophilic. Neutrophilic.

The mol% G + C of the DNA is: 59–62.

Type species: *Sulfitobacter pontiacus* Sorokin 1996, 362 (Effective publication: Sorokin 1995, 304.)

FURTHER DESCRIPTIVE INFORMATION

Ultrastructure Many cells of *Sulfitobacter pontiacus*, especially taken from batch culture, display an unusual compartmentalized cell ultrastructure (Fig. BXII.α.81). At least some cells appear to possess a major cell compartment bounded by a single membrane and containing the cell's nucleoid as well as electron-dense ribosome-like particles. A less electron-dense cytoplasm surrounds the compartment, with a larger region of this material at one pole comprising a polar cap. Such organization appears in thin sections whether cells are either chemically fixed or cryofixed and processed via cryosubstitution. An indication of such a structure may also be apparent from negatively stained whole cells (Sorokin, 1995). This type of cell organization has been referred to as a pirellulosome, an organelle reported previously only in the genus *Pirellula* (Lindsay et al., 1997), a member of the order *Planctomycetales*, which is a distinct phylogenetic lineage within the domain *Bacteria*. Members of at least two genera of planctomycetes (*Pirellula* and *Gemmata*) display unusual compartmented cell organization (Fuerst and Webb, 1991; Fuerst, 1995). Pirellulosomes of *S. pontiacus* differ from those in *Pirellula* in that in cells of the latter a nucleoid is always clearly visible as a condensed fibrillar region even after cryosubstitution, whereas in *Sulfitobacter* a condensed nucleoid in the pirellulosome is not clearly apparent in cryosubstituted cells, but is revealed after chemical fixation. This absence of distinct nucleoid in cryosubstituted cells is typical of the limited model bacteria so far examined by cryosubstitution. The occurrence of membrane-bounded nucleoid-containing cell compartments of analogous organization in two phylogenetically widely separated members of separate divisions or phyla of the domain *Bacteria* suggests either retention of an ancient characteristic of cell organization within deeply divergent lineages or convergent evolution of an analogous structure with similar function.

Metabolism Although *Sulfitobacter* species are obligate heterotrophs, *S. pontiacus* possesses a unique ability to derive additional metabolic energy from sulfite oxidation under substrate-limiting conditions, thus belonging to a specific type of lithoheterotrophs. The high tolerance to sulfite has also been demonstrated for other *Sulfitobacter* species but whether or not they are capable of sulfite oxidation and sulfite-dependent lithoheterotrophy remains unclear.

Habitat Sulfur-oxidizing chemolithoheterotrophic bacteria of the type represented by *S. pontiacus* rather than the autotrophic thiobacilli were found to dominate the sulfate-forming population in the Black Sea. This can be explained by better adaptation of these versatile heterotrophs to the conditions of the redox layer of the Black Sea, where relatively diluted upward fluxes of reduced sulfur compounds meet downward fluxes of organic matter from the euphotic surface layer. Recently, it was reported that bacteria closely related to *Sulfitobacter* represent a substantial part of heterotrophic population in littoral marine environments (González and Moran, 1997; Pukall et al., 1999).

Sulfitobacter brevis was isolated from the hypersaline lake, Ekho Lake, in East Antarctica (Vestfold Hills). The salinity of this lake increases with depth, with the formation of interface layers. *Sulfitobacter mediterraneus* was isolated from a microcosm prepared with natural seawater from the Mediterranean Sea.

ENRICHMENT AND ISOLATION PROCEDURES

Sulfitobacter pontiacus, together with other strains of sulfate-producing chemolithoheterotrophic bacteria from the Black Sea, were enriched on mineral medium containing seawater and thiosulfate. Although pure cultures of such bacteria cannot be grown on synthetic mineral media and demand an organic carbon source, the low amount of organic matter present in seawater, in combination with inorganic energy source allows selection of chemolithoheterotrophs. Pure cultures can be successfully grown on A medium with the following composition (per liter): NaCl, 20.0 g; HEPES (N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]), 7 g; K₂HPO₄, 1.0 g; NH₄Cl, 0.5 g; MgSO₄·7H₂O, 1.0 g; CaCl₂·2H₂O, 0.2 g; sodium acetate, 2.8 g; sodium thiosulfate, 2.5 g; yeast extract, 0.5 g; trace elements solution (Pfennig and Lippert, 1966), 1 ml; final pH 7.8. Short-term preservation of the active cultures (<1 month) is possible by storage of agar slopes at 4°C. Best survival during long-term storage (6–12 months) was registered when concentrated cultures were kept at –70°C with glycerol (15% v/v final concentration).

For the enrichment of *Sulfitobacter brevis*, 50 ml of saline Ekho Lake water (salinity 15‰) was amended with a filter-sterilized solution of 12.5 mg/ml. Oxoid yeast extract and incubated at 15°C under dim light. Colonies were obtained on PYGV agar¹ containing 25‰ (v/v) artificial seawater.

Enrichment of *Sulfitobacter mediterraneus* was accomplished by preparing a microcosm filled with 300 liters of natural seawater collected from a depth of 1 m at a station (42° 31' N, 03° 11' E) located about 2400 m from Banyuls-sur-Mer, France (Mediterranean Sea) and filtered through a 200-µm nylon mesh. At various times, isolation was performed by spreading 0.1 ml of 1:10, 1:100, and 1:1000 dilutions of the seawater onto plates of Marine Agar 2216 (Difco). Between 30 and 50 colonies were selected from each sampling time. Isolates were preserved by freezing at –80°C in 40% glycerol or by storing at 20°C on marine agar.

1. PYGV medium consists of (per liter): Bacto peptone (Difco), 0.25; Bacto yeast extract (Difco), 0.25; Hutner's basal mineral salt solution HBM (Cohen-Bazire et al., 1957), 20 ml; and vitamin solution No. 6 (van Ert and Staley, 1971), 10 ml. The medium is solidified with 18 g agar. For salt-requiring organisms, the medium is supplemented with 25‰ artificial sea water (ASW; Lyman and Fleming, 1940).

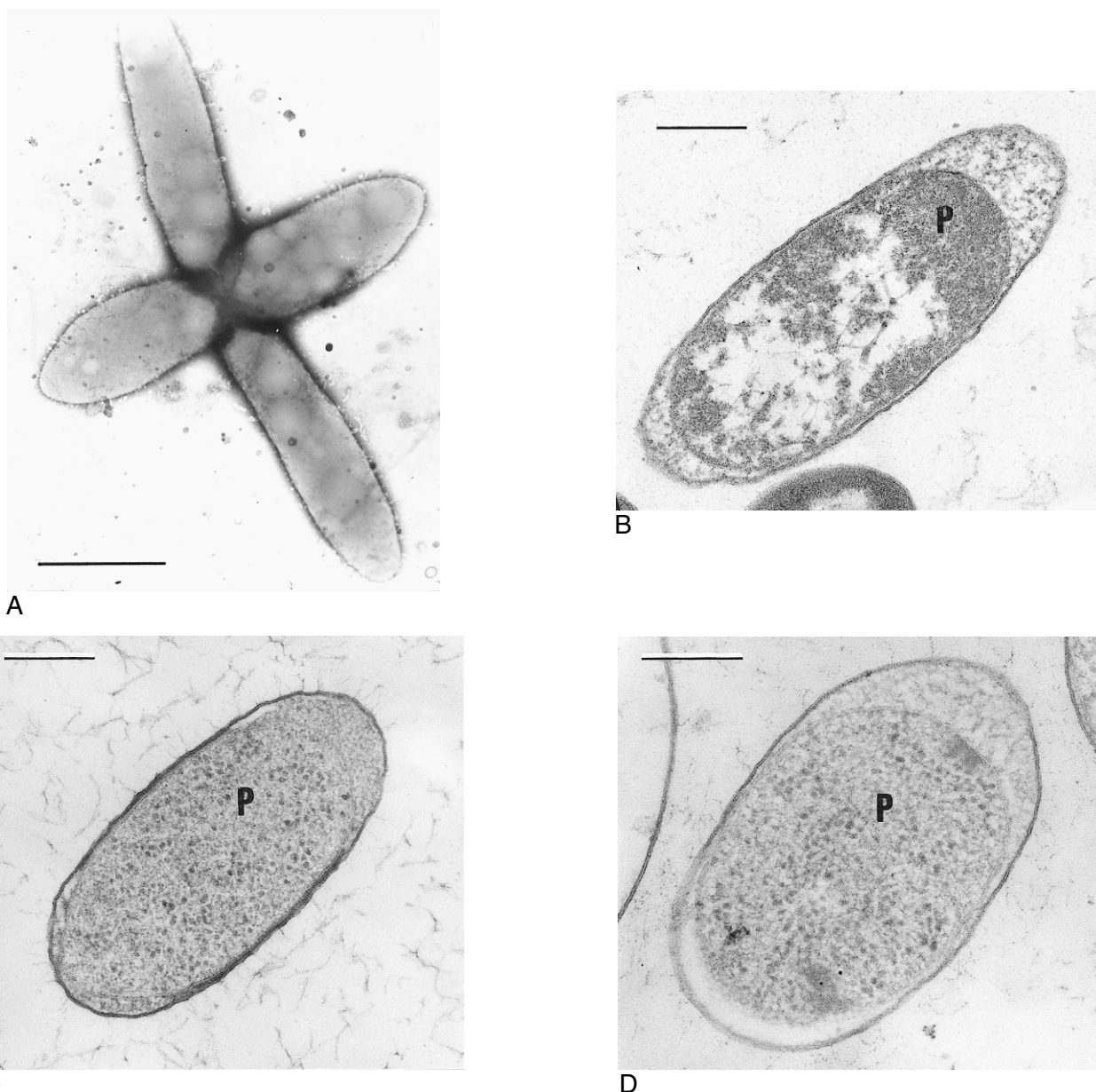


FIGURE BXII.α.81. Cell morphology of *Sulfitobacter pontiacus* (transmission electron micrographs). A–C, Strain CHLG 10. D, Strain CHLG 5. A, Total preparation stained with phosphotungstic acid. Bar = 1 μm . B, Thin-sectioned chemically fixed cell showing a major pirellulosome cell compartment (P) bounded by a single membrane and containing fibrillar nucleoid and electron-dense particulate cytoplasm and less electron-dense polar regions continuous with cytoplasm surrounding the membrane-bounded compartment. Bar = 0.1 μm . C–D, Thin-sectioned cryosubstituted cells, showing a major pirellulosome compartment (P) containing relatively large electron-dense particles bounded by a single membrane and a polar cap continuous with cytoplasm surrounding the pirellulosome. Bar = 0.1 μm .

DIFFERENTIATION OF THE GENUS *SULFITOBACTER* FROM OTHER GENERA

Table BXII.α.78 provides some characteristics that can be used to differentiate this genus from the closely related genera of the *Roseobacter* cluster.

TAXONOMIC COMMENTS

The original description of *Sulfitobacter pontiacus* was based on two closely related strains, which had a DNA–DNA reassociation value of about 90%: strain ChLG 10 (DSM 10014^T) and ChLG 5 (DSM 10015), isolated from the Black Sea (Sorokin, 1995). 16S rRNA gene sequence analysis revealed another representa-

tive of this species, strain SED3, isolated from the littoral waters near Australia (Ward-Rainey et al., 1996). Although different morphologically, this strain possesses a highly active sulfite dehydrogenase, typical for *Sulfitobacter*, and it showed a high percentage of DNA–DNA reassociation (>70%) with the type strain ChLG 10.

Phylogenetic analyses based on 16S rDNA sequence comparison demonstrate that *S. pontiacus* belongs to the *Alphaproteobacteria*, with its closest relatives being two of the three species of the aerobic bacteriochlorophyll-containing erythrobacteria of the genus *Roseobacter*, namely *Roseobacter litoralis* and *Roseobacter denitrificans* (Fig. BXII.α.82). The 16S rDNA sequence of *S. pontiacus* strain ChLG 10 has 96.7% and 97.5% similarity to the 16S

TABLE BXII.α.78. Differential characteristics of the genus *Sulfitobacter* and other phylogenetically related genera from the *Alphaproteobacteria*^a

Characteristics	<i>Sulfitobacter</i>	<i>Antarctobacter</i>	<i>Octadecabacter</i>	<i>Roseobacter</i> ^b	<i>Sagittula</i>	<i>Staleyia</i>
<i>Morphology:</i>						
Rosettes formation	+	+	—	—	+	+
Polar growth, pointed poles	+	+	—	—	+	+
Gas vesicles	—	—	+	—	—	—
<i>Physiology:</i>						
Sulfite oxidation	+	nd	nd	—	—	nd
Bchl <i>a</i>	—	—	—	+	—	+
Psychrophily	—	—	+	—	—	—
Denitrification	—	+	—	±	—	—
Gelatinase	—	+	—	+	—	—
Hydrolysis of cellulose and lignin	—	—	—	—	+	—
Biotin requirement	+	+	—	+	—	+
Mol% G + C of DNA	58–62	62.5	56–57	56–60	65	55–56.3

^aFor symbols see standard definitions; ±, variable; nd, not determined.^bBased on Bchl *a*-containing species.

rDNA sequences of *Roseobacter denitrificans* and *Roseobacter litoralis*, respectively. The only other complete 16S rDNA sequence with a high similarity to the *S. pontiacus* strain ChLG 10 sequence is that of strain S34, a marine bacterium isolated from the Sargasso Sea (Suzuki et al., 1997). With a 16S rDNA similarity value of 99.2% between the sequences of strain S34 and strain ChLG 10, it seems clear that strain S34 is a member of the genus *Sulfitobacter* and most probably an additional strain of the species *S. pontiacus*.

In the 16S rDNA sequence databases, there are additional partial (<500 nucleotides) sequences to which that of *S. pontiacus* (ChLG 10) shows greater than 98% sequence similarity. These sequences include those of the isolates EE-36 (99.7%), GAI-21 (98.8%) (González and Moran, 1997), and SED3 (99.3%) (Ward-Rainey et al., 1996). The high similarity between these sequences demonstrates the wide geographical distribution of strains of the genus *Sulfitobacter*.

The relatedness of *Sulfitobacter* to members of the genus *Roseobacter* is not surprising, considering that the class *Alphaproteobacteria* contains many species able to oxidize sulfur compounds, both photo- and chemotrophically. They are likely descendents of the purple nonsulfur bacteria, in particular of *Rhodobacter* spe-

cies. It could be speculated that colorless sulfate-forming lithoheterotrophs, like *S. pontiacus*, descended from the purple non-sulfur bacteria via erythrobacteria. In fact, some of the latter are capable of oxidizing reduced sulfur compounds (Yurkov et al., 1994b). Experiments with representatives of the genus *Roseobacter* demonstrated that the atypical *Roseobacter* species, *R. algicola*, was able to oxidize thiosulfate and sulfite to sulfate and possessed moderate sulfite dehydrogenase activity.

Analysis of nearly complete 16S rRNA gene sequences showed that the type strain of *S. brevis* was 96.5–98% similar to that of *S. pontiacus*. A high degree of sequence similarity occurred with *Roseobacter denitrificans* and *Roseobacter litoralis*. No significant DNA–DNA reassociation occurred between *S. brevis* and *S. pontiacus* (Labrenz et al., 2000).

16S rDNA sequence analysis of the type strain of *Sulfitobacter mediterraneus* indicated 98.2% similarity to the type strain of *S. pontiacus*. DNA–DNA reassociation experiments indicated a 46% hybridization value between the two species. Except for a quantitative difference in fatty acid composition, there are no distinct phenotypic differences between the two species (Pukall et al., 1999).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *SULFITOBACTER*

No clear-cut differences between the three species were found. The real differentiation was achieved based on DNA–DNA hybridization and 16S rDNA analysis. The presence of sulfite de-

hydrogenase and the ability to grow lithoheterotrophically with sulfur compounds remains to be tested for *S. brevis* and *S. mediterraneus*.

List of species of the genus *Sulfitobacter*

1. ***Sulfitobacter pontiacus*** Sorokin 1996, 362^{VP} (Effective publication: Sorokin 1995, 304.)
pon.ti.a'cus. Gr. n. *Pontus* Black Sea; M.L. adj. *pontiacus* from the Black Sea.

Cells grown in batch cultures vary in shape and size from regular rods (0.5–0.8 × 1.0–1.5 μm) to swollen filaments (up to 20 μm) and are very rarely motile. Bud-like minicells often appear in such cultures, especially when grown on solid medium. Cells grown in continuous culture are more regular, mostly motile rods with sharp edges, often arranged in rosettes (Fig.BXII.α.81a).

Sulfitobacter pontiacus benefits from the oxidation of reduced sulfur compounds such as thiosulfate and sulfite, being able to oxidize these to sulfate. This ability was proven in experiments with acetate-limited continuous culture (thiosulfate- or sulfite-dependent increase of specific

growth yield) and with washed cells (sulfite-dependent ATP production). An extremely active, AMP-independent, soluble sulfite dehydrogenase allows *S. pontiacus* to grow in the presence of very high concentrations of sulfite in acetate-limited continuous culture, which was not demonstrated previously even for chemolithoautotrophic sulfur-oxidizing bacteria. During cultivation, the bacterium gradually adapts to increasing sulfite concentrations and, at the same time, the specific biomass yield and the efficiency of acetate utilization increases.

Sole carbon and energy sources include acetate, propionate, butyrate, pyruvate, lactate, gluconate, acids of the Krebs cycle (except citrate and *cis*-aconitate), glycerol, glutamate, proline, aspartate, asparagine, serine, L-α-alanine, ornithine, and arginine as the sole carbon and energy sources. Ammonium salts and some amino acids are used

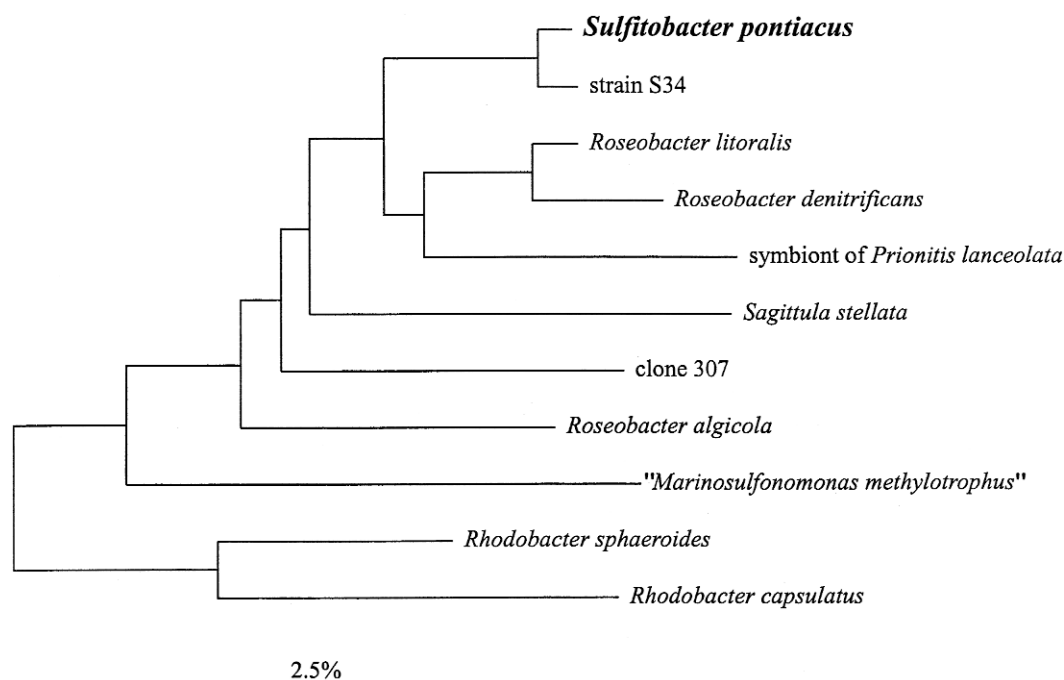


FIGURE BXII.α.82. Phylogenetic dendrogram based on comparison of 16S rDNA sequences indicating the position of *S. pontiacus* and its closest relatives within the *Alphaproteobacteria*. The dendrogram was reconstructed from evolutionary distances (Jukes and Cantor, 1969) by the neighbor-joining method (Saitou and Nei, 1987). Bar = 2.5 inferred nucleotide changes per 100 nucleotides. The accession numbers of the sequences used in these analyses are as follows: *Sulfitobacter pontiacus* (Y13155), strain S34 (U87407), *Roseobacter litoralis* (X78312), *Roseobacter denitrificans* (M59063), symbiont of *Prionitis lanceolata* (U37762), *Sagittula stellata* (U58356), clone 307 (U14583), *Roseobacter algicola* (X78315), "Marinosulfonomonas methylotrophus" (U62894), *Rhodobacter sphaeroides* (X53854), and *Rhodobacter capsulatus* (D16428).

as nitrogen sources. Growth on a mineral medium with acetate is stimulated by the presence of yeast extract and biotin. NaCl is required for growth; range, 5–80 g/l; optimal, 20–25 g/l). Temperature range for growth is 4–35°C (optimal, 22–25°C). The pH range for growth is 6.5–8.5 (optimal, 7.5–7.8).

No hydrolytic activities are present. Catalase and oxidase positive. Cytochromes c_{551} , b_{558} , and cytochrome c oxidase aa_3 are detectable in cell-free extracts. Isolated from the sulfide-oxygen interface water of the Black Sea.

The mol% G + C of the DNA is: 61.7–62.5 (T_m).

Type strain: ChLG 10, DSM 10014, VKM B-2022.

GenBank accession number (16S rRNA): Y13155.

2. ***Sulfitobacter brevis*** Labrenz, Tindall, Lawson, Collins, Schumann and Hirsch 2000, 311^{VP}
bre' vis. L. adj. *brevis* short, referring to the short cells.

Rods with one or both cell poles pointed, multiplying by monopolar growth, i.e., by a budding process. Cell sizes vary: $0.8\text{--}1.0 \times 1.1\text{--}1.5 \mu\text{m}$, with a mean size of $0.9 \times 1.3 \mu\text{m}$. Rosettes may be formed. Cells may be motile. Poly- β -hydroxybutyrate granules may be present. Endospores are not formed. On PYGV + ASW the colonies are smooth, convex, and yellowish brown. Do not grow photoautotrophically with H_2/CO_2 (80:20) or photoorganotrophically with acetate or glutamate. Peroxidase, catalase, and oxidase positive. Bacteriochlorophyll a is not produced.

Optimal growth temperature, 3–26°C. NaCl requirement, 1.0–2.0% NaCl or 10–80‰ ASW. Optimal pH for growth, 7.5–8.0. Cells have a requirement for pantothenate

and thiamin, a weak requirement for biotin and nicotinic acid, and none for vitamin B_{12} . Tween 80 is hydrolyzed, but not alginate, gelatin, DNA, or starch. Growth occurs on acetate, pyruvate, malate, succinate, citrate, butyrate, or glutamate, but not on methanesulfonic acid or methanol. α -D-Glucose is utilized weakly. Cells are susceptible to chloramphenicol, streptomycin, penicillin G, polymyxin B, and tetracycline, but not to nalidixic acid. Nitrate is not reduced. H_2S and indole are not produced. Methyl red and Voges-Proskauer negative.

Polar lipids present are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, and an unknown aminolipid. Dominant fatty acids are $\text{C}_{18:1\omega7\text{c}}$ and $\text{C}_{18:1\omega9\text{c}}$, and $\text{C}_{18:2}$ is present as two isomers. Other characteristic fatty acids are $\text{C}_{10:0\text{3OH}}$, $\text{C}_{14:1\text{3OH}}$, $\text{C}_{16:0}$, and $\text{C}_{18:0}$. Major respiratory quinone is Q-10. Source: water sample from Ekho Lake, Antarctica.

The mol% G + C of the DNA is: 57.9–58.1 (HPLC).

Type strain: EL-162, DSM 11443.

GenBank accession number (16S rRNA): Y16425.

3. ***Sulfitobacter mediterraneus*** Pukall, Buntetuß, Frühling, Rohde, Kroppenstedt, Burghardt, Lebaron, Bernard and Stackebrandt 1999, 518^{VP}
me.di.ter.ra.ne'us. N.L. adj. *mediterraneus* pertaining to the Mediterranean Sea.

Cells grown on marine agar at 25°C are rod shaped, $1\text{--}3 \times 0.5\text{--}0.8 \mu\text{m}$. Cells are motile by means of 1–5 subpolar flagella. Bacteria grown on marine agar, supplemented with acetate, tend to form rosettes and contain poly- β -hydroxy-

butyrate granules. Colonies on Marine agar are 1.2–1.4 mm in diameter, circular, convex, with entire or undulate margin, translucent and cream-colored. Temperature range for growth, 4–35°C; optimal, 17–28°C. pH range for growth, 6.5–8.5; optimal, 7.0–7.5. NaCl (2–80 g/l, optimal 15–20 g/l) is required for growth. Catalase and oxidase positive. Bacteriochlorophyll *a* is not present.

In minimal media with or without the addition of yeast extract or vitamins, growth occurs at 28°C with glucose, mannitol, gluconate, adipate, acetate, malate, pyruvate, lactate, propionate, butyrate, serine, proline, ornithine, alanine, asparagine, glutamate, and glycerol. Glutarate, *cis*-aconitate, tryptophan, caprate, urea, arabinose, and mannose are not utilized. Weak growth is detected with aspartate. Nitrate is not reduced. Utilization of citrate and maltose is stimulated in media containing yeast extract or biotin. Utilization of *N*-acetylglucosamine and activity of arginine

hydrolase, β -glucosidase, and gelatin hydrolase are induced after a longer incubation period (>3 d). Phenylacetate is used after 24 h incubation at 25°C. Growth occurs on 10 mM sulfite in acetate (10 mM)-supplemented HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[ethane-sulfonic acid]) medium. In TSB² medium containing 3% NaCl, the main fatty acid is *cis*-11 octadecenoic acid. Hexadecanoic acid occurs in smaller amounts. Signature nucleotides for 16S rDNA are located at positions 418–425 (C-G), 591–648 (C-G), 592–647 (A-U), and 599–639 (A-U). Isolated from coastal waters of the west Mediterranean Sea.

The mol% G + C of the DNA is: 59 (HPLC).

Type strain: CH-B427, DSM 12244.

GenBank accession number (16S rRNA): Y17387.

2. Tryptone Soya Broth was used for FAME analysis of *Sulfitobacter mediterraneus* and is available from Oxoid.

Genus Incertae Sedis XXVI. *Rhodothalassium* Imhoff, Petri and Süling 1998, 797^{VP}

JOHANNES F. IMHOFF

Rho'do.tha.las'si.um. Gr. n. *rhodon* the rose; Gr. adj. *thalassios* belonging to the sea; M.L. neut. n. *Rhodothalassium* the rose belonging to the sea.

Cells are vibrioid to spiral-shaped, motile by means of polar flagella and multiply by binary fission. **Gram-negative members of the *Alphaproteobacteria***. Internal photosynthetic membranes are present as lamellar stacks lying parallel to the cytoplasmic membrane. Photosynthetic pigments are **bacteriochlorophyll *a*** (esterified with phytol) and **carotenoids of the spirilloxanthin series**. **Ubiquinones and menaquinones with 10 isoprene units (Q-10 and MK-10) are present**. **Major cellular fatty acids are C_{18:1}, C_{18:0}, and C_{16:0}**, with C_{18:1} as dominant component (~60% of total fatty acids). **Cells grow preferably photoheterotrophically** under anoxic conditions in the light. **Chemotrophic growth is possible under oxic conditions in the dark**. Amino acids may be required as growth factors. **Obligately halophilic bacteria that require NaCl or sea salt for growth**, have salt optima above seawater salinity and an extended salt tolerance. Habitat: anoxic zones of hypersaline environments such as salterns and salt lakes that are exposed to the light.

The mol% G + C of the DNA is: 64.0.

Type species: *Rhodothalassium salexigens* (Drews 1982) Imhoff, Petri and Süling 1998, 797 (*Rhodospirillum salexigens* Drews 1982, 384.)

FURTHER DESCRIPTIVE INFORMATION

Rhodothalassium salexigens at present is the only representative of this genus. It is a moderately halophilic bacterium that requires elevated concentrations of salt for growth and is among the few extremely halotolerant species (up to 20% of salts) of the purple nonsulfur bacteria. Glycine betaine is accumulated as osmoticum and compatible solute in response to the external salt concentrations (Imhoff, 1992; Severin et al., 1992). Growth depends on glutamate as a growth factor or nitrogen source (Madigan et al., 1984). Cultures grown in the presence of 4 mM glutamate and dinitrogen form readily detectable amounts of nitrogenase (Madigan et al., 1984). The cell wall of *Rhodothalassium salexigens* contains peptidoglycan and proteins but lacks glycolipids and lipopolysaccharides (Golecki and Drews, 1980; Weckesser et al., 1995). Growth of *Rhodothalassium salexigens* is inhibited by a number of antibiotics such as tetracycline, chloramphenicol, penicil-

lin, ampicillin, cycloserine, nisin, vancomycin, and bacitracin, but not by oxacillin (Drews, 1981). Additional properties are given in Table BXII.α.79.

ENRICHMENT AND ISOLATION PROCEDURES

Rhodothalassium salexigens appears to be widely distributed in hypersaline environments such as salterns and salt lakes. Several strains of this species have been isolated from a saltern at the Mediterranean shore of Spain (Rodriguez-Valera et al., 1985). Standard techniques for isolation of purple nonsulfur bacteria in agar dilution series and on agar plates can be applied to *Rhodothalassium* species (Imhoff, 1988; Imhoff and Trüper, 1992). Media with neutral pH and elevated salt concentrations containing complex nutrients are suitable for their enrichment. A modification of the medium of Golecki and Drews (1980) has been successfully used and a recipe is given below (see also Imhoff, 1988).¹ *Rhodothalassium salexigens* also grows well on the complex medium of Nissen and Dundas (1984).

MAINTENANCE PROCEDURES

Cultures are well preserved by standard techniques in liquid nitrogen, by lyophilization or storage at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOTHALASSIUM* FROM OTHER GENERA

With respect to the natural environment, the obligate requirement for salt, cell morphology, and internal membrane structure, *Rhodothalassium* is clearly distinguished from other spiral-shaped phototrophic *Alphaproteobacteria*. In addition, it has a characteristic fatty acid and quinone composition and is clearly separated from other phototrophic purple bacteria by its 16S rDNA sequence. Major differentiating properties between *Rhodothalassium salexigens* and other phototrophic *Alphaproteobacteria* can be

1. The medium contains (per liter): 100 g NaCl, 3.5 g MgCl₂·6H₂O, 0.3 g KH₂PO₄, 10 mM sodium malate, 1.5 g yeast extract, 1.5 g protease peptone, and 1 ml trace element solution SLA. The initial pH is adjusted to 7.0.

TABLE BXII.α.79. Properties of *Rhodothalassium salexigens*^a

Characteristic	<i>Rhodothalassium salexigens</i>
Cell diameter (μm)	0.6–0.7
Internal membrane system	Lamellae
Motility	+
Color	Red
Bacteriochlorophyll	<i>a</i>
Growth factors	Glutamic acid
Aerobic growth	+
Oxidation of sulfide	–
Salt requirement	6–8% (5–20%)
Optimal temperature (°C)	40
Optimal pH	6.6–7.4
Habitat	Saltern
Mol% G + C of the DNA ^b	64.0
<i>Carbon sources utilized:</i>	
Acetate	+
Arginine	–
Citrate	+
Fructose	–
Glucose	+
Glutamate	+
Glycerol	+
Lactate	–
Pyruvate	+
Succinate	+
<i>Electron donors utilized:</i>	
Sulfide	–
Sulfur	–
Thiosulfate	–
Major quinones	Q-10, MK-10
<i>Major fatty acids:</i>	
C _{14:0}	3.8
C _{16:0}	16.1
C _{16:1}	1.5
C _{18:0}	17.8
C _{18:1}	59.9

^aSymbols: +, positive in most strains; –, negative in most strains; Q-10, ubiquinone 10; MK-10, menaquinone 10.

^bDetermined by buoyant density centrifugation.

seen by comparing Table BXII.α.1 in the chapter for the genus *Rhodospirillum* with Table BXII.α.79. Carbon sources used can be compared between Table BXII.α.2 in the chapter for the genus *Rhodospirillum* and Table BXII.α.79. The phylogenetic relationship of these bacteria based on 16S rDNA sequences is presented in Fig. 1 (p. 124) in the introductory chapter on “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A.

List of species of the genus *Rhodothalassium*

- Rhodothalassium salexigens*** (Drews 1982) Imhoff, Petri and Söling 1998, 797^{VP} (*Rhodospirillum salexigens* Drews 1982, 384.)
sal.ex'i.gens. L. n. *sal* salt; L. part. adj. *exigens* demanding; M.L. adj. *salexigens* salt-demanding.

Cells are rod-shaped to spiral, 0.6–0.7 × 1–6 μm; one complete turn of a spiral is 0.8–0.9 μm wide. Internal photosynthetic membranes occur as lamellae lying parallel to the cytoplasmic membrane. Color of anaerobically grown liquid cultures is red. Absorption maxima of living cells are at 375, 485, 515, 550, 590, 800, 840, and 875 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spirilloxanthin series with spirilloxanthin as the predominant component. Cells grow photoheterotrophically under anoxic conditions in the light with various organic compounds as carbon and

TAXONOMIC COMMENTS

Because of its spiral shape and phototrophic capacity, *Rhodothalassium salexigens* was originally assigned to the genus *Rhodospirillum*, which comprised all spiral-shaped purple nonsulfur bacteria at that time (Drews, 1981). High diversity among these bacteria was revealed by their chemical composition in regard to fatty acids and quinones, but also in respect to the structure of internal membrane systems, to growth factor requirement and salt dependency. Because of outstanding phenotypic differences and the great genetic distance from the type species of this genus, *Rhodospirillum rubrum*, and to other purple nonsulfur bacteria, it was reclassified as *Rhodothalassium salexigens* (Imhoff et al., 1998).*

FURTHER READING

- Imhoff, J.F., R. Petri and J. Söling. 1998. Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the *Alpha-proteobacteria*: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomense* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. Int. J. Syst. Bacteriol. 48: 793–798.
- Imhoff, J.F. and H.G. Trüper. 1992. The genus *Rhodospirillum* and related genera. In Balows, Trüper, Dworkin, Harder and Schleifer (Editors), The Prokaryotes: A Handbook on the Biology of Bacteria. Ecophysiology, Isolation, Identification, Applications, Springer-Verlag, New York. pp. 2141–2155.
- Kawasaki, H., Y. Hoshino and K. Yamasato. 1993. Phylogenetic diversity of phototrophic purple non-sulfur bacteria in the alpha proteobacteria group. FEMS Microbiol. Lett. 112: 61–66.

*Editorial Note: Based on 16S rDNA sequence information, *Rhodothalassium salexigens* forms a distinct phylogenetic lineage, separate from other so-called “purple non-sulfur bacteria.” This separation is so significant that it precludes inclusion of the genus *Rhodothalassium* in any of the families of the *Alphaproteobacteria*. Though its phylogenetic position would allow *Rhodothalassium* to be considered a separate family, 16S rDNA sequence evidence is only available from a single strain. Therefore, further data should be obtained prior to assigning this genus to a family. At present, the genus *Rhodothalassium* is considered a *genus incertae sedis* with distant affiliations to the families *Rhodospirillaceae*, *Rhodobacteraceae*, and *Rhizobiaceae*.

electron sources or under oxic conditions in the dark. Carbon compounds assimilated are shown in Table BXII.α.79. Photoautotrophic growth with molecular hydrogen, sulfide, or thiosulfate as electron donor is not possible. Ammonia or dinitrogen cannot serve as sole nitrogen source; casein hydrolysate is used as a carbon and nitrogen source. Glutamate is required for growth, but no other growth factors are required. Obligately and moderately halophilic bacterium with optimal growth at 40°C (range 20–45°C), pH 6.6–7.4, and 6–8% NaCl (range: 5–20% NaCl). No growth occurs in the absence of salt. Habitat: anoxic zones of hypersaline environments such as salterns and partially evaporated pools of seawater with decaying plants that are exposed to the light.

The mol% G + C of the DNA is: 64.0 (Bd, *T_m*).

Type strain: WS 68, ATCC 35888, DSM 2132.

GenBank accession number (16S rRNA): D14431.

Order IV. *Sphingomonadales* ord. nov.

EIKO YABUUCHI AND YOSHIMASA KOSAKO

Sphing.o.mon.a.da'les. M.L. fem. n. *Sphingomonas* type genus of the order; *-ales* ending to denote an order; M.L. fem. pl. n. *Sphingomonadales* the order of *Sphingomonas*.

Gram-negative, nonsporeforming rod-shaped, ovoid, or pleomorphic cells. Reproduction is usually by binary fission, but in some members, polar growth or budding has been observed by electron microscopy. Motile or nonmotile. **Instead of lipopolysaccharide (LPS) in cell wall, glucuronosyl ceramide (SGL-1) and 2-OH myristic acid are present, without 3-OH acids.** Instead of C_{14:0 2OH} **nonhydroxy myristic acid in *Zymomonas mobilis*. Aerobic,** having a strictly respiratory type of metabolism with oxygen as

the terminal electron acceptor, with the exception of the genus *Zymomonas*, which is **facultatively anaerobic and has a strictly fermentative type of metabolism.** Some species synthesize bacteriochlorophyll *a* and are **facultative phototrophs. Chemoheterotrophic. The major respiratory quinone is ubiquinone Q-10** in those genera that have been tested. Belong to the class *Alpha-proteobacteria*. Free living and widely distributed in nature.

TABLE BXII.α.80. 16S rDNA sequence homology ratios of 49 type strains of 48 species in 13 genera of *Alphaproteobacteria* to *Sphingomonas paucimobilis* EY 2395^T

Order ^a	Family	Genus	Species	Type strains used	Sequence data accession number	% Similarity to GIFU 2395 ^T
IV	I	I	<i>Sphingomonas paucimobilis</i>	GIFU ^b 2395 ^T	D16144	100
IV	I	I	<i>S. parapaucimobilis</i>	JCM 7510 ^T	X72721	97.7
IV	I	I	<i>S. sanguinis</i>	IFO 13937 ^T	D13726	97.7
IV	I	I	<i>S. roseiflava</i>	MK341 ^T	D84520	96.8
IV	I	I	<i>S. adhaesiva</i>	GIFU 11458 ^T	D16146	96
IV	I	I	<i>S. pituitosa</i>	EDIV ^T	AJ243751	95.8
IV	I	I	<i>S. trueperi</i>	LMG 2142 ^T	X97776	95.5
IV	I	I	<i>S. aquatilis</i>	KCTC 2881 ^T	AF131295	95.4
IV	I	I	<i>S. koreensis</i>	KCTC 2882 ^T	AF131296	95.4
IV	I	I	<i>S. echinoides</i>	ATCC 14820 ^T	AB021370	94.7
IV	I	I	<i>S. asaccharolytica</i>	IFO 10564 ^T	Y09639	94.3
IV	I	I	<i>S. mali</i>	IFO 10550 ^T	Y09638	94.1
IV	I	I	<i>S. melonis</i>	DAPP PG-244 ^T	AB055563	94
IV	I	I	<i>S. pruni</i>	IFO 15498 ^T	Y09637	93.9
IV	I	I	<i>S. wittichii</i>	RW1 ^T	AB021492	93.6
IV	I	I	<i>S. rosa</i>	IFO 15208 ^T	D13945	92.5
IV	I	I	<i>S. aromaticivorans</i>	IFO 16084 ^T	AB25014	92.3
IV	I	I	<i>S. yanoikuyae</i>	GIFU 9882 ^T	D16145	92.1
IV	I	I	<i>S. cloacae</i>	S-3 ^T	AB040739	92.1
IV	I	I	<i>S. suberifaciens</i>	IFO 15211 ^T	D13737	91.9
IV	I	I	<i>S. xenophaga</i>	BN6 ^T	X94098	91.9
IV	I	I	<i>S. subterranea</i>	IFO 16086 ^T	AB25012	91.8
IV	I	I	<i>S. ursincola</i>	KR-99 ^T	Y10677	91.8
IV	I	I	<i>S. subarctica</i>	KF-3 ^T	X94103	91.7
IV	I	I	<i>S. taejonensis</i>	KCTC 2884R	AF131297	91.7
IV	I	I	<i>S. chlorophenolica</i>	ATCC 33790 ^T	X87161	91.4
IV	I	I	<i>S. macrogoltabidus</i>	IFO 15033 ^T	D13723	91.4
IV	I	I	<i>S. stygia</i>	IFO 16085 ^T	AB25013	91.4
IV	I	I	<i>S. capsulata</i>	GIFU 11526	D16147	91.3
IV	I	I	<i>S. herbicidovorans</i>	Not recorded	AB042233	91.2
IV	I	I	<i>S. natatoria</i>	DSM 3183 ^T	Y13774	90.7
IV	I	I	<i>S. terrae</i>	IFO 15098 ^T	D13727	90.2
IV	I	I	<i>S. alaskensis</i>	RB2256 ^T	Z73631	90.9
IV	I	I	<i>S. chungbukensis</i>	DJ77 ^T	AF129257	90.8
IV	I	VI	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	ATCC 10988 ^T	RDP ^c	90.4
IV	I	VI	<i>Zymomonas mobilis</i> subsp. <i>pomaceae</i>	ATCC 29192 ^T	RDP	90.4
IV	I	III	<i>Erythromicrobium ramosum</i>	E5 ^T	X72909	92.1
IV	I	II	<i>Erythrobacter litoralis</i>	T4 ^T	X72962	91.9
IV	I	V	<i>Sandaracinobacter sibiricus</i>	RB16-17 ^T	Y10678	91.8
IV	I	IV	<i>Porphyrobacter tepidarius</i>	OT3 ^T	D84429	91.3
IV	I	II	<i>Erythrobacter longus</i>	OCH 101 ^T	M96744	90.7
IV	I	IV	<i>Porphyrobacter neustonensis</i>	ACM 2844 ^T	L01785	90.6
I	I	I	<i>Rhodospirillum rubrum</i>	ATCC 11170 ^T	D30778	85.2
I	I	II	<i>Acetobacter aceti</i>	DSM 3508 ^T	X94066	84.3
II	I	I	<i>Rickettsia prowazekii</i>	Not recorded	M21789	83.4
III	I	I	<i>Rhodobacter capsulatus</i>	ATCC 11166 ^T	D16428	84.8
V	I	I	<i>Caulobacter subvibrioides</i>	ATCC 15264 ^T	AB008392	85.6
V	I	III	<i>Brevundimonas diminuta</i>	DSM 1635 ^T	X87274	87
VI	I	I	<i>Rhizobium leguminosarum</i>	IAM 12609 ^T	D14513	87.6

^aOrder I. *Rhodospirillales*^{AL}; II. *Rickettsiales*^{AL}; III. *Rhodobacterales*; IV. *Sphingomonadales*; V. *Caulobacterales*^{AL}; VI. *Rhizobiales*.

^bSame strain as EY 2395^T.

^cRDP, Ribosomal Data Project.

The mol% G + C of the DNA is: 59–68.5; (*Z. mobilis*, 49.1).

Type genus: *Sphingomonas* Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321 (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116) emend. Takeuchi, Hamana and Hiraishi 2001, 1414; emend. Yabuuchi, Kosako, Fujiwara, Naka, Matsunaga, Ogura and Kobayashi 2002, 1489.

FURTHER DESCRIPTIVE INFORMATION

If motile, motility is by means of polar or subpolar-monotrichous, polar-multitrichous, or peritrichous flagella.

In addition to SGL-1, galacturonosyl ceramide (SGL-1') is detected in several species. SGL-1 is commercially used to moisturize human skin.

No data are available concerning the quinones of *Zymomonas mobilis*.

Strains of some species biodegrade dibenzo-*p*-dioxin and related compounds. Cells that are aggregated or attached to goethite (α -FeOOH) particles inactivate chlorine (Gauthier et al., 1999). Some extracellular polysaccharides, e.g., sphingane (Pollock, 1993), are of industrial use.

Strains of the genera have been isolated from a variety of environments, including chemically contaminated soil, subsurface sediment, river water, seawater, alkaline spring, hot springs, distilled water, sewage, plants, hospitals, or found in association with diseased plant and human hosts. The wide variety of isolation sources is reflected in the nutritional diversity of the organisms.

Circumscription of the Order Yabuuchi et al. (1990a) transferred the species *Pseudomonas paucimobilis* Holmes et al. 1977a

(previously known as CDC group IIK-2) to a new genus, *Sphingomonas*, as the type species, *Sphingomonas paucimobilis*. Eventually, the genus was expanded to include 34 species. These were later included in a new family, *Sphingomonadaceae*, by Kosako et al. (2000a). This family is presently the only family in the order *Sphingomonadales* and contains nine genera.

The percent similarity of the 16S rDNA oligonucleotide sequences of the various members of *Sphingomonadales* to that of the type species of the genus *Sphingomonas* ranges from 90.4% to 97.7%. The percent similarity of the 16S rDNA of five other orders in the *Alphaproteobacteria* ranges from 83.4% to 87.6% (Table BXII.α.80). Analysis of the 16S rDNA sequences indicates a close relationship of *Zymomonas mobilis* to the genus *Sphingomonas* (Fig. BXII.α.83). Signature nucleotide sequences of the 16S rDNA (Table BXII.α.81) and the dendrogram of six type species of *Sphingomonadaceae* (Fig. BXII.α.84) indicate the base of construction of this order. The mol% G + C of the DNA ranges from 59 to 68.5, with the exception of *Z. mobilis*, which has a value of 49.1.

It is unlikely that levels of 16S rDNA sequence similarity or the presence or absence of specific cellular components can define the order. It is now apparent that phenotypic characteristics such as flagellar morphology, fermentation vs. nonfermentation of glucose, fermentative vs. respiratory metabolism, and (as mentioned by Kondratieva et al., 1992) the synthesis of bacteriochlorophyll *a* cannot be used as a key characteristic to exclude any genus or family from the order. It is possible that development of new, easy-to-use identification methods based on molecular techniques such as DNA-tips might be able to accurately differentiate the taxa within the order.

TABLE BXII.α.81. Signature sequences of 16S rDNA oligonucleotides among the members of Class *Alphaproteobacteria*

Order	Species	Position						
		108–116	433–442	750–754	822–825	855–858	1334–1336	1385
IV	<i>S. paucimobilis</i> and 32 <i>Sphingomonas</i> sp.	CACGGGTGC	GCTCTTTTAC	TTGAC	ATAA	GGCG	GGC	A
	<i>S. stygia</i>	CACGGGTGC	GCTCTTTTAC	[CTGAC] ^a	ATAA	GGCG	GGC	A
	<i>Erythrobacter longus</i>	CACGGGTGC	GCTCTTTTAC	TTGAC	ATAA	GGCG	GGC	A
	<i>Erythrobacter litoralis</i>	CACGGGTGC	GCTCTTTTAC	TTGAC	ATAA	GGCG	* * * ^b	*
	<i>Erythromicrobium ramosum</i>	CACGGGTGC	GCTCTTTTAC	TTGAC	ATAA	GGCG	* * *	*
	<i>Sandaracinobacter sibiricus</i>	CACGGGTGC	GCTCTTTTAC	TTGAC	[ACGA]	GGCG	GGC	A
	<i>Z. mobilis</i>	CACGGGTGC	GCTCTTTTAC	TTGAC	ATAA	GGCG	GGC	A
I	<i>Rhodospirillum rubrum</i>	CACGGGTGA ^c	GCTCTTTTCGG	TTGAC	AGTG	GTCG	GTC	G
I	<i>Acetobacter aceti</i>	GACGGGTGA	GCACITTTTCGG	CTGAC	TGTG	GTCG	GGT	G
II	<i>Rickettsia prowazekii</i>	GACGGGTGA	GCTCTTTTAG	CTGAC	AGTG	TTCG	GTT	G
III	<i>Rhodobacter capsulatus</i>	GACGGGTGA	GCTCTTTTCAG	CTGAC	ATGC	GACA	GTT	G
V	<i>Caulobacter subvibrioides</i>	* * * * GGTGA	ATACTTTCAC	CTGAC	ATTG	GACG	GTT	G
V	<i>Brevundimonas diminuta</i>	GACGGGTGA	ATTCTTTCAC	CTGAC	ATTG	GACG	GTT	G
VI	<i>Rhizobium leguminosarum</i>	GACGGGTGA	GCTCTTTCAC	CTGAC	AATG	GGCG	GTT	G
	<i>Escherichia coli</i> JO-1	GACGGGTGA	GCTCTTTCAG	CTGAC	TCCA	TCCG	GTC	G

^a[], Sequence containing nucleotide(s) different from those of *S. paucimobilis*.

^b*, Defect of nucleotide.

^cGothic letter: nucleotide different from that of organism in Order IV.

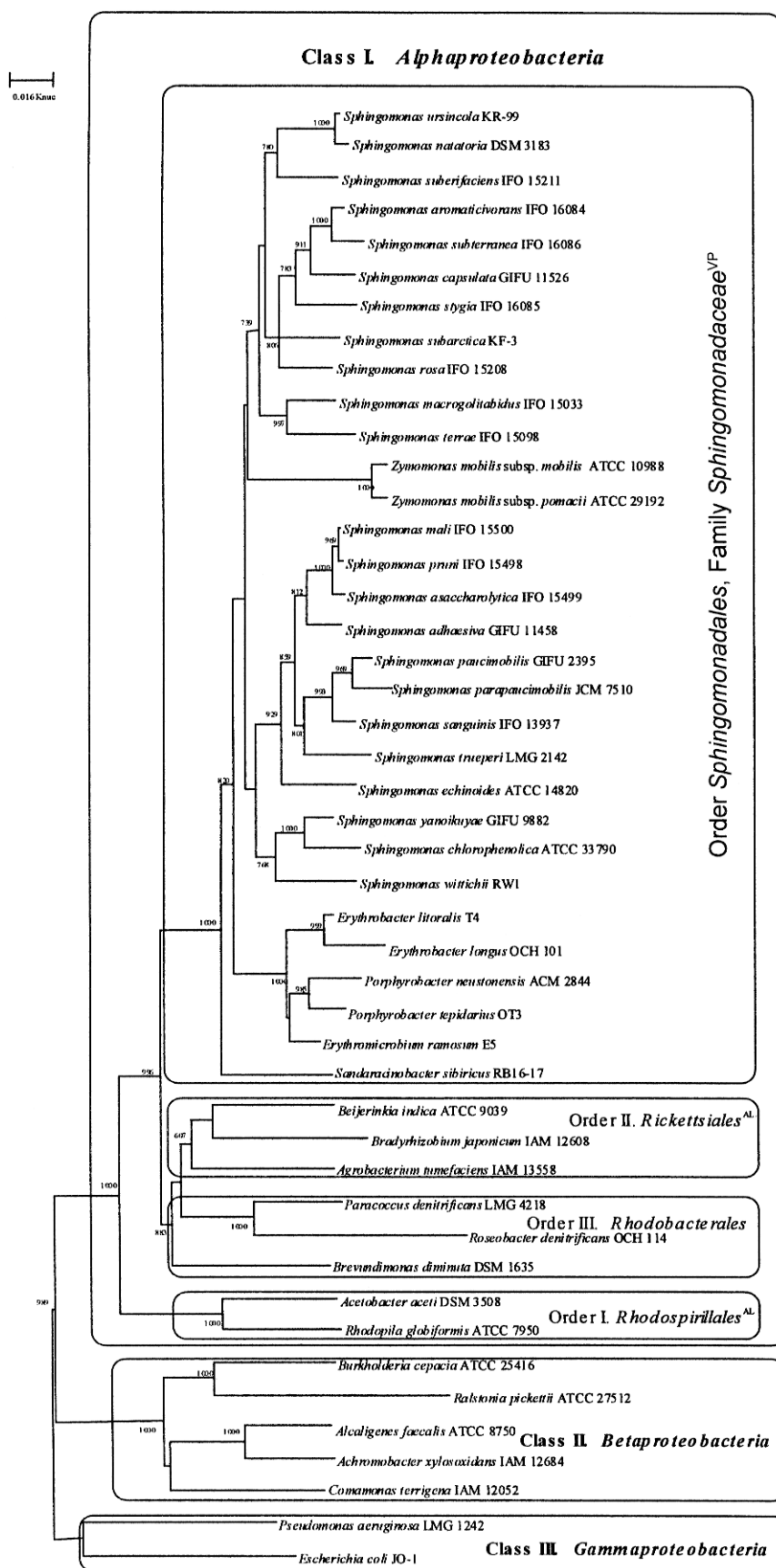


FIGURE BXII.α.83. Phylogenetic position of *Sphingomonadales*, *Sphingomonadaceae*, and *Sphingomonas* among the members of *Proteobacteria* obtained by the neighbor-joining analysis (Saitou and Nei, 1987) of 16S rDNA. Scale bar = 16 nucleotide substitutions per 1000 nucleotides of the 16S rDNA sequence. Boot strap values from 1000 analyses are shown at the branch points. Class I. *Alphaproteobacteria*. Order I. *Rhodospirillales*. Order II. *Rickettsiales*. Order III. *Rhodobacterales*. Order IV. *Sphingomonadales*. Class II. *Betaproteobacteria*. Class III. *Gammaproteobacteria*.

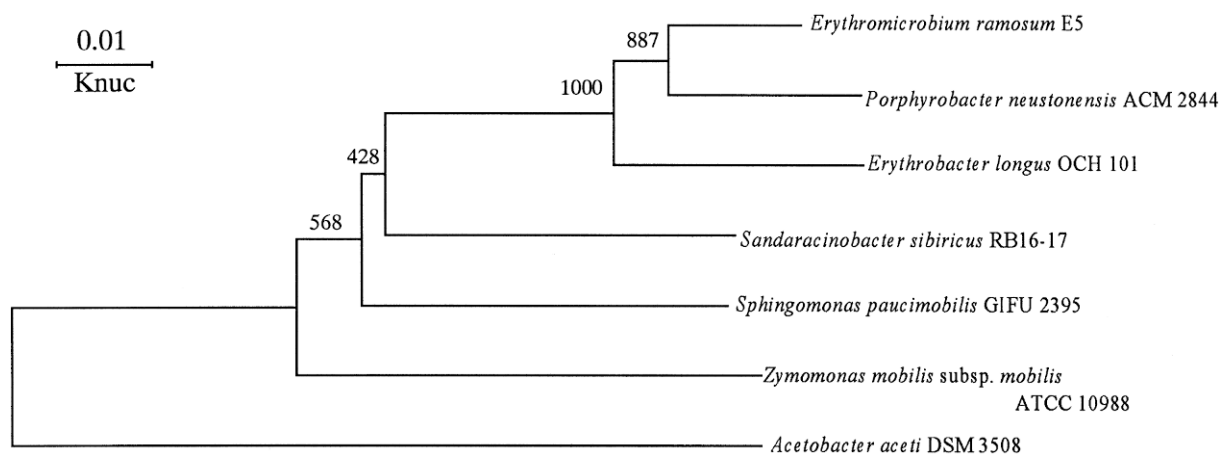


FIGURE BXII.α.84. Phylogenetic position of type species of six genera of family *Sphingomonadaceae* obtained by the neighbor-joining analysis of 16S rDNA. *Acetobacter aceti* was used as an out group species.

Family I. **Sphingomonadaceae** Kosako, Yabuuchi, Naka, Fujiwara and Kobayashi 2000b, 1953^{VP} (Effective publication: Kosako, Yabuuchi, Naka, Fujiwara and Kobayashi 2000a, 563)

EIKO YABUUCHI AND YOSHIMASA KOSAKO

Sphing.o.mon.a.da'ceae. M.L. fem. n. *Sphingomonas* type genus of the family; *-aceae* ending to denote an family; M.L. fem. pl. n. *Sphingomonadaceae* the family of *Sphingomonas*.

The characteristics are as given for the order. Differential characteristics of the genera in the family *Sphingomonadaceae* are listed in Table BXII.α.82.

The mol% G + C of the DNA is: 59–68.5 (*Z. mobilis*, 49.1).

Type genus: **Sphingomonas** Yabuuchi, Yano, Oyaizu, Hashi-

moto, Ezaki and Yamamoto 1990b, 321 (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116) emend. Takeuchi, Hamana and Hiraishi 2001, 1414; emend. Yabuuchi, Kosako, Fujiwara, Naka, Matsunaga, Ogura and Kobayashi 2002, 1489.

TABLE BXII.α.82. Differential characteristics of the genera in the family *Sphingomonadaceae*^a

Characteristics	<i>Sphingomonas</i>	<i>Erythrobacter</i>	<i>Erythromicrobium</i>	<i>Porphyrobacter</i>	<i>Sandaracinobacter</i>	<i>Zymomonas</i>
Cell diameter, μm	0.2–1.4	0.5–0.7	0.6–1.0	0.5–0.7	0.3–0.5	1.0–1.2
Cell length, μm	0.5–4.0	0.7–5.0	1.3–2.5	0.8–1.4	1.5–2.5	2.0–6.0
Strictly respiratory type of metabolism	+	+	+	+	+	–
Glucuronosyl ceramide present	+	+	+	+	nd	+
Galacturonosyl ceramide present	D	–	–	–	nd	–
2-OH myristic acid present	+	+	+	+	nd	–
Bacteriochlorophyll <i>a</i>	D	+	+	+	+	–
Facultative phototrophic	D	+	+	+	+	–
Major carotenoid	Nostoxanthin ^b	Zeaxanthin ^c	Zeaxanthin ^c	Carotenoid sulfate	Carotenoids ^d	–
Oxidase	D	+	+	–	+	–
Catalase	+	+	+	+	–	+
Growth factor requirement	D	+	+	–	nd	+
Fermentation of glucose	–	–	–	–	–	+
Colony color	Orange, yellow, white, colorless	Brown to red	Orange	Orange to red	Yellow to orange	–
Major respiratory quinone	Q-10	Q-10	Q-10	Q-10	Q-9, Q-10	nd
NaCl requirement	–	+	–	–	–	–
Mol% G + C of DNA	59–67.2	63.6–66	63.6–64.2	66.4–65.0	68.5	47.7–48.5

^aSymbols: +, positive; –, negative; D, different reaction in different species; nd, not determined.

^bData from Jenkins et al. (1979).

^cData from Hanada et al. (1997).

^dData from Yurkov et al. (1997).

Genus I. **Sphingomonas** Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116) emend. Takeuchi, Hamana and Hiraishi 2001, 1414; emend. Yabuuchi, Kosako, Fujiwara, Naka, Matsunaga, Ogura and Kobayashi 2002, 1489

EIKO YABUUCHI AND YOSHIMASA KOSAKO

Sphin.go.mo' nas. Gr. gen. n. *sphingos* of sphinx; Gr. n. *monad* unit, monad; M.L. fem. n. *Sphingomonas* a sphingosine-containing monad.

Straight or slightly curved rods or ovoid cells 0.2–1.4 × 0.5–4.0 µm. Gram negative. Asporogenous. Reproduction in most species is by binary fission; budding or asymmetric division as visualized by electron microscopy occurs in two species. Motile or nonmotile. A rosette-like aggregation caused by polar fimbriae occurs in some species. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Anaerobic nitrate respiration does not occur. Esculin is hydrolyzed. **Two species with bacteriochlorophyll *a* are facultative photoorganotrophs.** Colony color varies from orange or yellow to white to nonpigmented. Catalase positive. Oxidase positive or negative. **Glucuronosyl-(1→1)-ceramide (SGL-1), galacturonosyl-β(1→1)-ceramide (in several species), and 2-hydroxymyristic acid occur, but not 3-hydroxy fatty acids** (Table BXII.α.83, Figs. BXII.α.85, BXII.α.86, and BXII.α.87). The lipopolysaccharide (LPS) of the cell wall is replaced by sphingolipids (Figs. BXII.α.88 and BXII.α.89). C_{18:1 ω9t} and C_{18:1 ω7c} are the major nonpolar fatty acids and **2-hydroxymyristic acid** is the major 2-hydroxy acid (Table BXII.α.84). Some species are opportunistic pathogens, causing meningitis, septicemia, peritonitis, and neonatal infections in intensive care units. Pathogenicity toward animals is not known. **Free living** in natural and man-made environments.

The mol% G + C of the DNA is: 59–68.

Type species: *Sphingomonas paucimobilis* (Holmes, Owen, Evans, Malnick and Wilcox 1977a) Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321 (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116) (*Pseudomonas paucimobilis* Holmes, Owen, Evans, Malnick and Wilcox 1977a, 133.)

FURTHER DESCRIPTIVE INFORMATION

Cellular and flagellar morphology Cell sizes range from 0.4 × 1.4 µm in *S. paucimobilis* to 0.7–1.2 × 1.3–4.0 µm in *S. xenophaga*. By Gram staining, the cells of the type strains of most species are straight or slightly curved rods with rounded ends, whereas those of *S. echinoides* have sharp ends (Stolz et al., 2000). Electron micrographs of *S. natatoria* and *S. ursincola* have revealed a budding type of reproduction, but this has not been seen in photomicrographs of Gram-stained preparations. A rosette-like arrangement was reported in *S. paucimobilis* (Yabuuchi et al., 1979), *S. natatoria* (*B. natatorius* Sly 1985), and *S. echinoides* Heumann and Marx (1964) described the star (rosette)-formation of the organism and illustrated the phenomenon by electron micrographs. Cells aggregated or attached to goethite (α-FeOOH) particle inactivate chlorine (Gauthier et al., 1999). The aggregation is caused by attachment and irreversible contraction of the monopolar fimbriae of two or more cells.

Diffuse spreading growth does not occur on 0.3% semisolid agar plates in *S. capsulata* and 13 other species. These are recognized as nonmotile. Peritrichous flagellation of *S. trueperi* EY4218^T is shown in Fig. BXII.α.90.

Colony characteristics Colonies on agar plating media suitable for each species are pinpoint in size after 48 h or more incubation at 25–30°C. Colonies are approximately 1 mm in diameter when fully grown, circular, domed, and smooth with entire margins. Colonies are pigmented at the beginning of their appearance on agar media. Members of the genus, except the type strain of *S. herbicidovorans*, do not produce water-soluble pigments. Pigmentation of colonies differs from species to species

TABLE BXII.α.83. Distribution of the five molecular species of sphingoglycolipid (SGL) among the members of *Sphingomonadaceae*^a

SGL on TLC	Species	EY No.	Long-chain base(s)	Carbohydrate moiety(ies)	Hydroxy fatty acid(s)
1	<i>S. paucimobilis</i>	2395 ^T	C _{18:0} , C _{20:1} , C _{21 cyclo}	Glucuronic acid	C _{14:0} 2OH
	<i>S. capsulata</i>	4216 ^T	C _{18:0} , C _{20:1} , C _{21 cyclo}	Glucuronic acid	C _{14:0} 2OH
	<i>S. yanoikuyae</i>	4208 ^T	C _{20:1} , C _{21 cyclo}	Glucuronic acid	C _{14:0} 2OH
	<i>Erythrobacter longus</i>	4203 ^T	C _{20:1}	Glucuronic acid	C _{14:0} 2OH, C _{15:0} 2OH, C _{16:0} 2OH
	<i>S. natatoria</i>	4220 ^T	C _{19:1} , C _{20:1}	Glucuronic acid	C _{14:0} 2OH, C _{15:0} 2OH, C _{16:0} 2OH
	<i>S. cloacae</i>	4361 ^T	nd	Glucuronic acid	nd
	<i>Zymomonas mobilis</i>	4209 ^T	C _{16:0}	Glucuronic acid	(C _{14:0} , C _{16:0}) ^b
1'	<i>S. yanoikuyae</i>	4208 ^T	C _{20:1} , C _{21 cyclo}	Galacturonic acid	C _{14:0} 2OH
	<i>S. terrae</i>	4207 ^T	nd	Galacturonic acid	nd
	<i>S. macrogoltabidus</i>	4304 ^T	nd	Galacturonic acid	nd
	<i>S. wittichii</i>	4224 ^T	nd	Galacturonic acid	nd
	<i>S. cloacae</i>	4361 ^T	nd	Galacturonic acid	nd
2	<i>S. paucimobilis</i>	2395 ^T	nd	Glucosamine, glucuronic acid	nd
3	<i>S. capsulata</i>	4216 ^T	C _{18:0} , C _{20:1} , C _{21 cyclo}	Galactose, glucosamine, glucuronic acid	C _{14:0} 2OH
4	<i>S. paucimobilis</i>	2395 ^T	C _{18:0} , C _{20:1} , C _{21 cyclo}	Mannose, galactose, glucosamine, glucuronic acid	C _{14:0} 2OH
	<i>S. sanguinis</i>	2397 ^T	nd	nd	nd
	<i>S. parapaucimobilis</i>	4213 ^T	nd	nd	nd

^aAbbreviations: cyclo, cyclopropanoic acid; nd, not determined.

^bNonhydroxy acid.

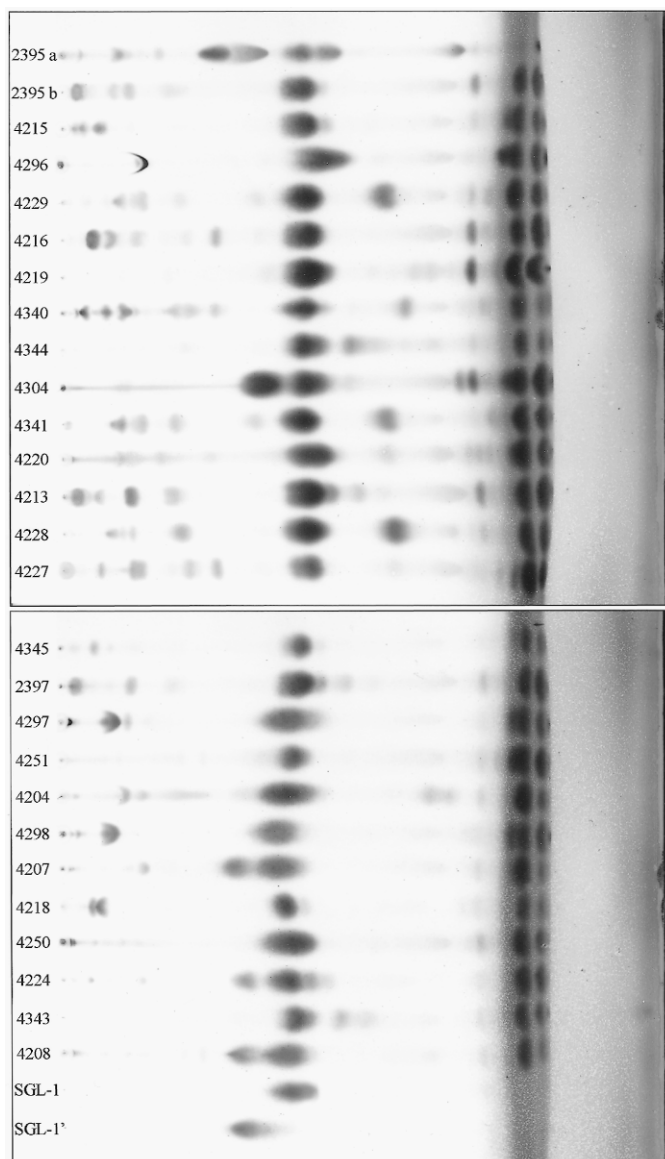


FIGURE BXII.α.85. Thin-layer chromatograms of alkali-stable lipids of type strains of 26 *Sphingomonas* species. EY strain number and species, from top to bottom: 2395^T, *S. paucimobilis* (a = crude lipids; b = alkali-stable lipids); 4215^T, *S. adhaesiva*; 4296^T, *S. aromaticivorans*; 4229^T, *S. asaccharolytica*; 4216^T, *S. capsulata*; 4219^T, *S. chlorophenolica*; 4340^T, *S. echinoides*; 4344^T, *S. herbicidovorans*; 4304^T, *S. macrogoltabidus*; 4341^T, *S. mali*; 4220^T, *S. natatoria*; 4213^T, *S. parapaucimobilis*; 4228^T, *S. pruni*; 4227^T, *S. rosa*; 4345, *S. roseiflava*; 2397, *S. sanguinis*; 4297^T, *S. stygia*; 4251^T, *S. subarctica*; 4204^T, *S. suberifaciens*; 4298^T, *S. subterranea*; 4207^T, *S. terrae*; 4218^T, *S. trueperi*; 4250^T, *S. ursincola*; 4224^T, *S. wittichii*; 4343^T, *S. xenophaga*; 4208^T, *S. yanoikuyae*; purified SGL-1, purified SGL-1'. Solvent system: chloroform/methanol/acetic acid/H₂O (100:20:12:5, by vol).

and may be influenced by the medium and time of incubation. A characteristic deep-yellow color is due to the carotenoid nostoxanthin (Jenkins et al., 1979), rather than the brominated aryl-polyamine xanthomonadin that is produced by *Xanthomonas* sp. (Starr et al., 1977; Jenkins and Starr, 1985). Colonies of some initially nonpigmented strains such as *S. yanoikuyae* become lemon yellow after 3 days incubation at room temperature. The type strain of *S. herbicidovorans* produces the water-soluble brown pigment alcapton by accumulation of homogentisic acid in pep-

tone media (Table BXII.α.85), as observed in the pyromelanogenic strain of *Pseudomonas aeruginosa* (Mann, 1969; Yabuuchi and Ohya, 1972; Gessard, 1981). The type strain of *S. herbicidovorans* represents the second confirmed bacterium with a defective homogentisicase, which results in accumulation of homogentisic acid. The mechanism is similar to that in the human inborn error, alcaptonuria.

Sphingoglycolipids The presence of sphingolipids among the cells of eucaryotes and procaryotes is well known. Sphingolipids are divided into sphingophospholipids and sphingoglycolipids. The skeleton of sphingolipids is ceramide, which is composed of sphingosine (long chain base coupled with a fatty acid molecule by acidic amide linkage). Sphingomyelin is one of the most common mammalian sphingophospholipids. Sphingophospholipids are the characteristic component of the cellular lipids of *Sphingobacterium* spp. and *Prevotella* spp. Molecular species of sphingoglycolipids (SGLs) are differentiated according to their number (1 to 4) and kinds of carbohydrates (glucuronic acid, glucosamine, galactose, and mannose). These carbohydrates are linked to the alcoholic hydroxyl base by a glycosidic bond. Both glucuronosyl-ceramide (SGL-1) (Yamamoto et al., 1978) and galacturonosyl-ceramide (SGL-1') (Naka et al., 2000) are novel sphingoglycolipids among procaryotes and eucaryotes. The chemical structure, function, and distribution of SGL of *Sphingomonas* species in *Proteobacteria* have been reported to replace lipopolysaccharide in the cell wall of Gram-negative rods (Kawahara et al., 1990, 1991, 1999, 2000; Kawasaki et al., 1994c).

A key enzyme in sphingolipid biosynthesis, serine palmitoyl-transferase (SPT, EC 2.3.1.50), has been successfully purified. When the gene encoding 420 amino acid residues was overexpressed in *Escherichia coli*, the recombinant SPT was indistinguishable from the native enzyme, both catalytically and spectrophotometrically (Ikushiro et al., 2001). *Sphingomonas* SPT is recognized as a prototype of the eucaryotic enzyme, and an investigation of the sphingolipid biosynthetic enzymes of *Sphingomonas* would help to clarify sphingolipid biosynthesis of both procaryotes and higher organisms (Ikushiro et al., 2001).

Localization of SGL-4 of the type strain of *S. paucimobilis* has been visualized by gold-labeled immunoelectron microscopy (Fig. BXII.α.88), and a model of the outer membrane of *S. paucimobilis* is shown in Fig. BXII.α.89. Stimulation of phagocytosis and phagosome-lysosome fusion in human neutrophils was reported (Miyazaki et al., 1995). Induction and release of monokine by SGL of type strain of *S. paucimobilis* by different mechanisms from those of LPS were reported (Tahara and Kawazu, 1994; Krziwon et al., 1995).

Cellular fatty acid composition The presence of 2-hydroxy-myristic acid and the lack of 3-hydroxy acid of any kind in cellular lipid are important characteristics of members of *Sphingomonadaceae* (except the anaerobic species *Zymomonas mobilis*) (Fig. BXII.α.86). Minor components may change their concentration because of culture conditions and are not useful as differential characters among species.

The fatty acid α-hydroxylase of the type strain of *S. paucimobilis* was partially purified, and direct involvement of hydrogen peroxide in the α-hydroxylation was confirmed (Fig. BXII.α.91). Interestingly, molecular oxygen was not required for α-hydroxylation if hydrogen peroxide was present (Matsunaga et al., 1996). The nucleotide sequence of the fatty acid α-hydroxylase gene was deposited in DDBJ/EMBL/GenBank with accession number AB006957 (Matsunaga et al., 1998), and the deduced amino acid sequence of the enzyme is shown in Fig. BXII.α.92. The

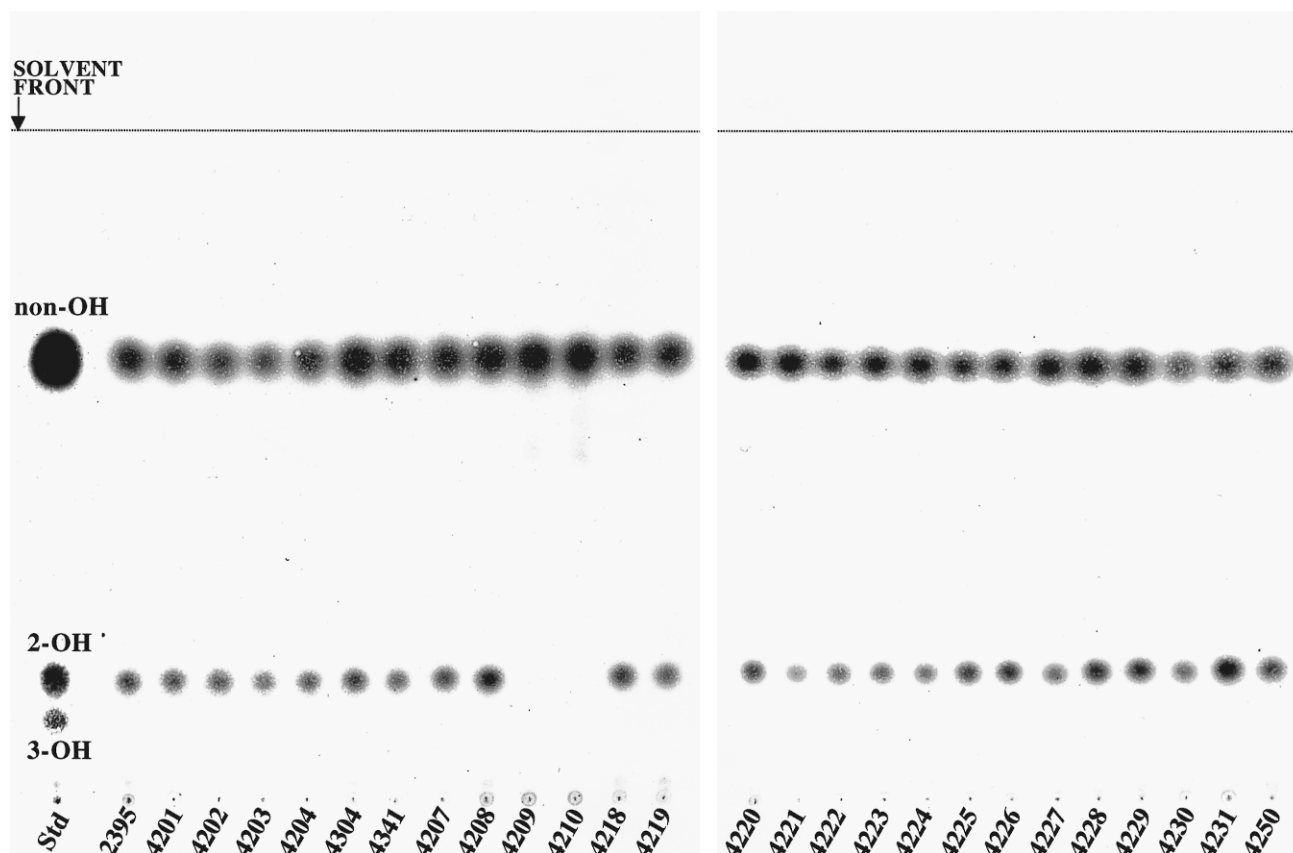


FIGURE BXII.α.86. Thin-layer chromatograms of cellular fatty acids of 21 type and 5 reference strains of member species of family *Sphingomonadaceae*. Std = standard, EY strain number and species, from left to right: 2395^T = *S. paucimobilis*; 4201 = *S. yanoikuyae*; 4202 = *S. yanoikuyae*; 4203^T = *Erythrobacter longus*; 4204^T = *S. suberifaciens*; 4304^T = *S. macrogoltabidus*; 4341^T = *S. mali*; 4207^T = *S. terrae*; 4208^T = *S. yanoikuyae*; 4209^T = *Zymomonas mobilis*; 4210^T = *Z. mobilis* subsp. *pomacii*; 4218^T = *S. trueperi*; 4219^T = *S. chlorophenolica*; 4220^T = *S. natatoria*; 4221 = *S. chlorophenolica*; 4222^T = *Erythrobacter litoralis*; 4223^T = *Erythromicrobium ramosum*; 4224^T = *S. wittichii*; 4225 = *Sphingomonas* sp. SS3; 4226 = *S. yanoikuyae* B1; 4227^T = *S. rosa*; 4228^T = *S. pruni*; 4229^T = *S. asaccharolytica*; 4230^T = *Porphyrobacter neustonensis*; 4231^T = *Porphyrobacter tepidarius*; 4250^T = *S. ursincola*. Solvent system: *n*-hexane/diethyl ether (80:20, by vol). Nonhydroxy acid and no 3-hydroxyacid in all 26 strains. 2-hydroxy acid in 24 strains with two exceptions of *Zymomonas* strains. (Chromatogram reproduced from Y. Kosako et al., *Microbiology and Immunology*, 44: 563–575, 2000, ©Japanese Society for Bacteriology, Tokyo.)

fatty acid α -hydroxylase gene was defined as a cytochrome P450 enzyme and overexpressed in *Escherichia coli* (Matsunaga et al., 1997).

Biodegradation of dibenzo-*p*-dioxin and related compounds

Soil and water pollution by polychlorinated derivatives of dibenzo-*p*-dioxin (DD) has become a problem worldwide. In addition to the most toxic compound 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), there are many possible congeners, as shown in Fig. BXII.α.93. Accordingly, numerous articles have been published concerning the aerobic degradation pathways (Fig. BXII.α.94) used by strain RW1 of *Sphingomonas wittichii*—the best-known metabolizer of DD and DF (dibenzofuran) (Wittich et al., 1992; Bünz and Cook, 1993; Bünz et al., 1993; Happe et al., 1993; Moore et al., 1993; Bertini et al., 1995; Thakur, 1996; Arfmann et al., 1997; Armengaud and Timmis, 1997, 1998; Megharaj et al., 1997; Armengaud et al., 1998, 1999; Keim et al., 1999; Leung et al., 1999; Vuilleumier et al., 2001). Bioremediation of environments polluted with halogenated DDs and DFs seems difficult because of inefficient attack on the highly halogenated congeners by the initial dioxygenase of RW1 and because of the lack of catabolic pathways for the mineralization of halogenated intermediates.

Other species of *Sphingomonas* are capable of degrading a huge range of recalcitrant compounds of environmental concern. *S. paucimobilis* can degrade biphenyl (Davison et al., 1996, 1999) and utilize the herbicide diclofop-methyl (Smith-Greenier and Adkins, 1996), γ -hexachlorocyclohexane (Miyauchi et al., 1998), and aromatic hydrocarbons (Shuttleworth et al., 2000). *S. yanoikuyae* can degrade (polycyclic) aromatic hydrocarbons (Klecka and Gibson, 1980; Eaton et al., 1996b; Khan et al., 1996a; Kim et al., 1997; Lloyd-Jones and Lau, 1997; Kim and Zylstra, 1999; Kazunga and Aitken, 2000; Shuttleworth et al., 2000). *S. subarctica* degrades chlorophenols (Nohynek et al., 1996a); *S. aromaticivorans*, *S. subterranea*, and *S. stygia* degrade aromatic hydrocarbons (Balkwill et al., 1997); *S. herbicidovorans* degrades Mecoprop (Zipper et al., 1996); *S. chlorophenolica* degrades pentachlorophenol (Nohynek et al., 1995, 1996a; McCarthy et al., 1997; Ohtsubo et al., 1999; Xu et al., 1999; Xun et al., 1999); and *S. xenophaga* degrades naphthalene sulfonic acids and *N,N*-dimethylaniline (Stolz et al., 2000).

Numerous *Sphingomonas* isolates are capable of degrading diphenyl ethers (Schmidt et al., 1992a, b, 1993), dibenzofuran, and substituted dibenzofurans (Fortnagel et al., 1990; Harms and Zehnder, 1994; Harms et al., 1995; Wittich et al., 1999), 2,4-D

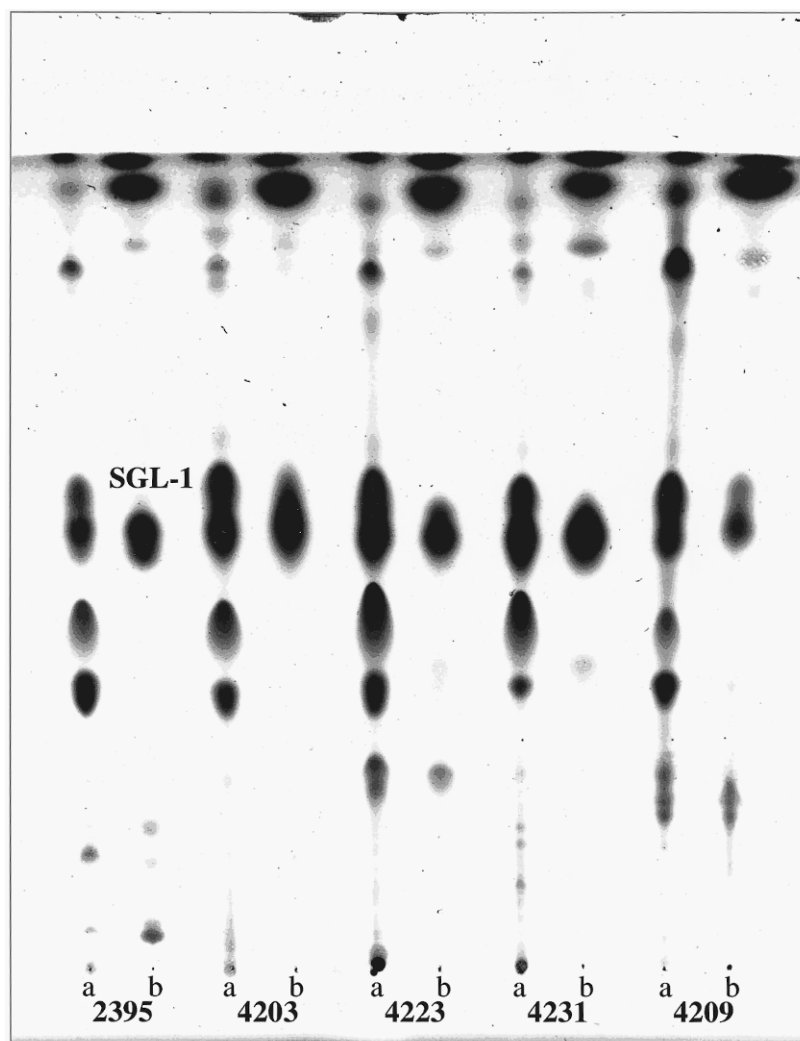


FIGURE BXII.α.87. Thin-layer chromatograms of cellular lipids and alkali-stable lipids of type strains in the family *Sphingomonadaceae*. EY strain number and species, from left to right: 2395^T = *S. paucimobilis*; 4203^T = *Erythrobacter longus*; 4223^T = *Erythromicrobium ramosum*; 4209^T = *Zymomonas mobilis*. Solvent system: chloroform/methanol/acetic acid/H₂O (100:20:12:5, by vol).

(Ka et al., 1994a, b, c), (polycyclic) aromatic hydrocarbons (Fredrickson et al., 1995; Dagher et al., 1997; Ederer et al., 1997; Harayama, 1997; Mueller et al., 1997), aromatics and chloroaromatics (Yrjala et al., 1998), pentachlorophenol (Karlson et al., 1995), gentisate (Werwath et al., 1998), 4-methylquinoline (Pfaller et al., 1999), and carbofuran (Ogram et al., 2000).

Strain RW1 previously adapted to DD- and DF-contaminated soil was found to survive better in DD- and DF-amended soil and biodegrade DD and DF more efficiently than bacteria that had not been pre-adapted (Megharaj et al., 1997). There are some other interesting reports on biodegradation of compounds of environmental concern in polluted soil by strains of this genus (Thomas et al., 2000) and utilization of herbicide dichlorofop-methyl by soil microorganism as the sole source of carbon and energy (Smith-Greenier and Adkins, 1996). The use of pentachlorophenol-degrading sphingomonads for environmental clean up was reported (Colores et al., 1995) and may be exploited in the future.

Extracellular heteropolysaccharide Production of agar-like polysaccharide by *Pseudomonas* species was reported by Kang et al. (1982). Gellan S-60 (Kang and Veeder, 1982a), welan S-130 (Kang and Veeder, 1982b), rhamsan, S-88 (Kang and Veeder, 1985), S-198, and S-657 are members of a family of microbial polysaccharides (Moorhouse, 1987). The structure of these Gellan-related heteropolysaccharides was called "sphingan" by Pollock (1993), who recognized the producers as members of the genus *Sphingomonas*. The biochemical functions of the glycosyl transferase genes essential for biosynthesis of exopolysaccharides have been reported by Pollock et al. (1998). A heteropolysaccharide isolated from a culture of a "*Pseudomonas elodea*" strain is a repeating linear tetrasaccharide composed of D-glucose, D-glucuronic acid, and L-rhamnose in a ratio 2:1:1 (Jansson et al., 1983); side chains were not present. An α-rhamnosidase of *Sphingomonas* sp. R1 has been reported (Hashimoto and Murato, 1998). X-ray crystallographic analysis and genetic studies were reported for the alginate lyases of a strain of *Sphingomonas* sp.

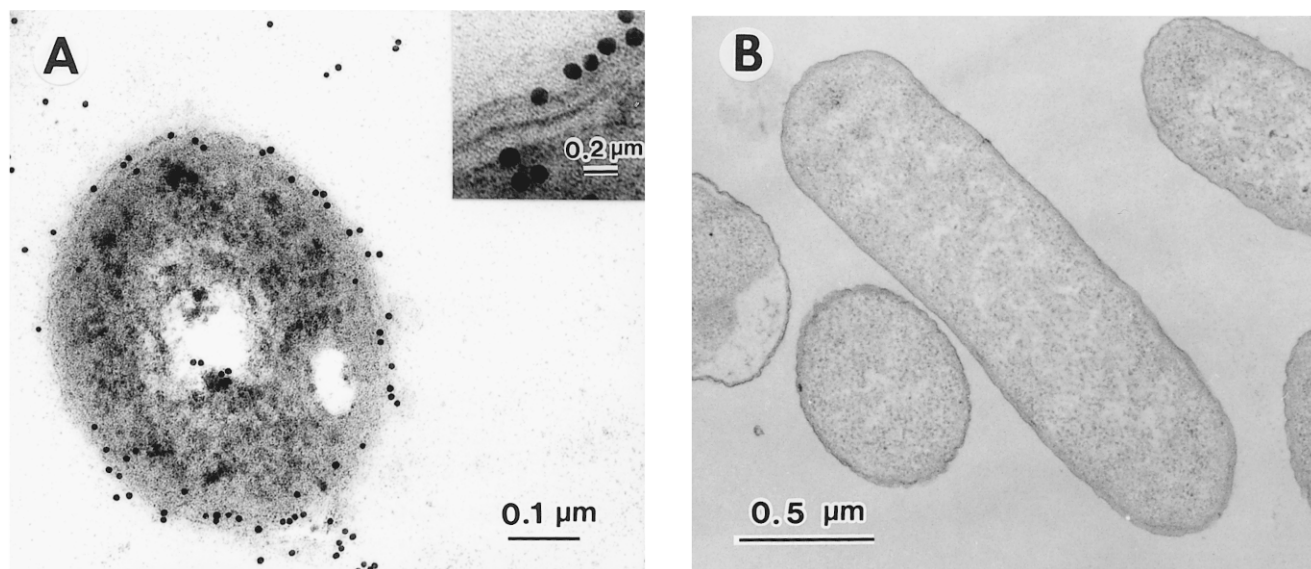


FIGURE BXII.α.88. Localization of sphingoglycolipid on the outer membrane of *Sphingomonas paucimobilis*. (A) *Sphingomonas paucimobilis* IAM 12576^T, immunogold-labeled soma after embedding. Ultrathin-sectioned cell was first treated with anti-SGL-4A antibody, and then stained with gold-labeled secondary antibody. Primary antibody recognized SGL-4A alone, not SGL-1. Gold particles observed on entire surface of the soma, but none on (B) *Escherichia coli* used as control. (Reproduced with permission from S. Kawasaki et al., *Journal of Bacteriology* 176: 284–290, 1994, ©American Society for Microbiology.)

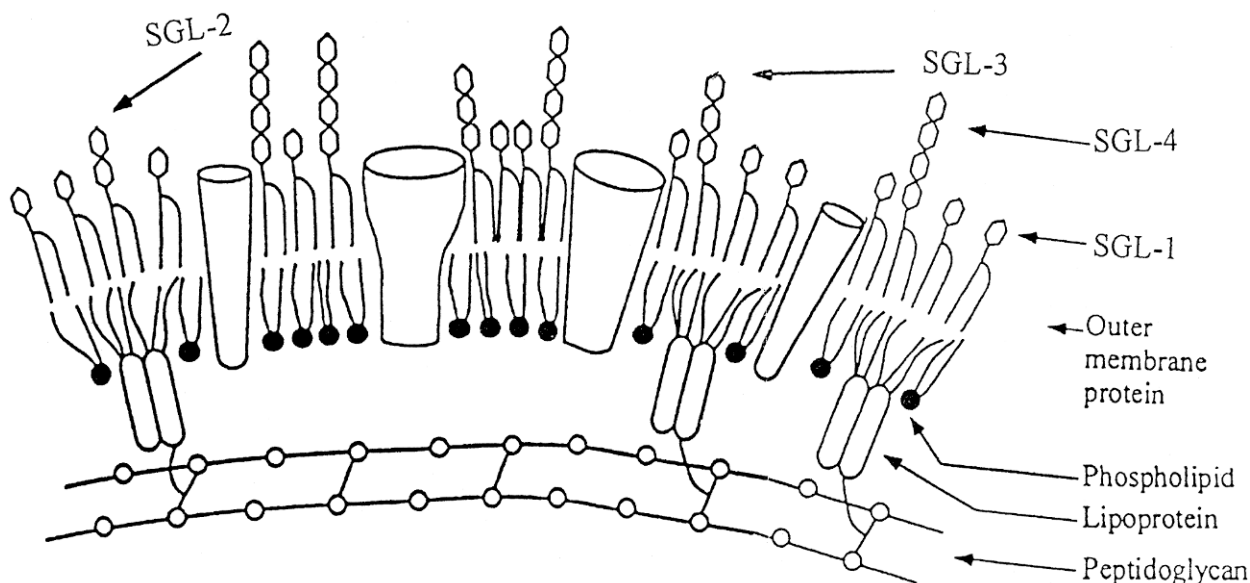


FIGURE BXII.α.89. Outer membrane model of *Sphingomonas paucimobilis*. SGL-1 glucuronic acid–Ceramide; SGL-2 glucosamine–glucuronic acid–Ceramide; SGL-3 galactose–glucosamine–glucuronic acid–Ceramide; SGL-4 = mannose–galactose–glucosamine–glucuronic acid–Ceramide. (Modified with permission from K. Kawahara et al., *Journal of Industrial Microbiology and Biotechnology* 23: 408–413, 1999, ©Specialist Journals Division, Nature Publishing Group.)

(Yoon et al., 2000a, b). A *pgmG* gene, which encodes a bifunctional enzyme in Gellan gum-producing *S. paucimobilis*, was reported by Videira et al. (2000).

Industrial usage of the heteropolysaccharides has been reported (Peik et al., 1983, 1985), and modified heteropolysaccharides might possibly be used for oil recovery (Robinson and Stipanovic, 1989).

Ecological features Sphingomonads are free living in natural and man-made environments. Yellow-pigmented aerobic Gram-negative rods have often been isolated from polluted and un-

polluted environments and identified as members of the genus *Sphingomonas*. Furthermore, many yellow-colored isolates formerly assigned to the genus *Flavobacterium*, and others earlier classified as *Pseudomonas* and possibly *Moraxella*, might belong to the genus *Sphingomonas*, but they have not been reinvestigated to date.

Metabolic features The presence of either spermidine or homospermidine differs among species. Assimilation tests for selected organic compounds can serve to differentiate *Sphingomonas* species (Table BXII.α.86). Esculin is hydrolyzed, although

TABLE BXII.α.84. Cellular fatty acid composition (%) of the type strains of 39 species and 2 subspecies in the family *Sphingomonadaceae*^{a,b}

Fatty acids, % of total	Species and Strains																				
	<i>Sphingomonas paucimobilis</i> EY 2395 ^T	<i>Sphingomonas adhaesiva</i> EY 4215 ^T	<i>Sphingomonas alaskensis</i> EY 4374 ^T	<i>Sphingomonas aquatilis</i> KCTC 2881 ^{Tc}	<i>Sphingomonas aromaticivorans</i> EY 4296 ^T	<i>Sphingomonas asaccharolytica</i> EY 4229 ^T	<i>Sphingomonas capsulata</i> EY 4216 ^T	<i>Sphingomonas chlorophenolica</i> EY 4219 ^T	<i>Sphingomonas chungbukensis</i> EY 4375 ^T	<i>Sphingomonas cloacae</i> EY 4361 ^T	<i>Sphingomonas echinoides</i> EY 4340 ^T	<i>Sphingomonas herbicidovorans</i> EY 4344 ^T	<i>Sphingomonas korensis</i> EY 4376 ^T	<i>Sphingomonas macrogoltabidus</i> EY 4304 ^T	<i>Sphingomonas mali</i> EY 4341 ^T	<i>Sphingomonas melonis</i> EY 4350 ^T	<i>Sphingomonas natatoria</i> EY 4220 ^T	<i>Sphingomonas parapaucimobilis</i> EY 4213 ^T	<i>Sphingomonas pituitosa</i> EY 4370 ^T	<i>Sphingomonas pruni</i> EY 4228 ^T	<i>Sphingomonas rosea</i> EY 4227 ^T
C _{14:1}																					
C _{14:0}	1	1		1	tr		tr		tr	tr		tr	tr	tr	tr	1		2	tr		tr
C _{15:0 iso}					2						14										
C _{15:0}		1	3	nd	tr	tr	tr						tr		1			tr	2		
C _{16:1 ω7c}	2	4	3		16		2	7	5	3	1	5	1	29	tr	4	5	3	1		17
C _{16:1}		1	1	1	1	1	1	1	3	2		1	3	3			1	tr	2		tr
C _{16:0}	17	13	3	20.6	3	4	7	9	8	4	8	6	7	10	14	9	6	7	12	10	9
C _{17:1}			9	nd	5	3									3						
C _{17:0 cyclo}		4	40		12	32	2	tr	4			1	4	tr	20	2	1	2	9	3	tr
C _{17:0}		1	3	nd	1	9	tr	tr	tr		4		tr		6	tr		tr	tr		tr
C _{18:2}																					
C _{18:1 ω9t}	60	55	11	61	43	19	66	66	65	69	41	67	61	43	45	51	65	61	49	55	60
C _{18:1 ω7c}																					
C _{18:1}		tr			tr	tr	2	1		4		2	3	tr	2	tr	1	3	tr	2	tr
C _{18:0}	1	tr	3	1	2	tr	2	tr	tr	4	2	tr	tr	tr	tr	5	1	2	4	1	
C _{19:1}						tr															
C _{19:0 cyclo}	trb					1															
C _{19:0}						tr					2										
C _{14:0 2OH}	18	18	2	10.9	10	3	16	10	14	13	23	14	17	7	5	26	8	17	20	14	11
C _{15:0 2OH}			21	nd	4	15	tr											tr	2		
C _{16:1 2OH}																	6				
C _{16:0 2OH}				nd			tr	1				3	3	4	3		4	tr		2	
C _{14:0 3OH}																					
C _{16:0 3OH}																					
Other	tr	tr	1	5		11		3		tr	5			3		1	2	tr	tr	11	1
Total	99	98	100	101	99	97	98	98	99	99	100	99	99	99	99	99	100	97	99	100	98

^aHydrolysis was carried out in HCl-methanol(1:5,v/v), 100°C, 3 h.^bAbbreviations: tr, <1.0%; cyclo, cyclopropanoic acid; nd, not determined.^cData from Lee et al. (2001).

prolonged incubation may be needed for a positive reaction by some species. Additional information can be found in Nohynek et al. (1996a), Kämpfer et al. (1997), Denner et al. (1999), Stolz et al. (2000), and Denner et al. (2001).

Antibiotic sensitivity Of 33 type strains tested, 91–100% were susceptible to tetracyclines, amikacin, gentamicin, panipenem, and imipenem (Table BXII.α.87). Differences in susceptibilities to amoxicillin (67%) and amoxicillin/clavulanic acid (91%) suggest the presence of penicillinase in these species (Table BXII.α.87).

Pathogenicity *Sphingomonas* species can act as opportunistic pathogens (Decker et al., 1992) and have caused meningitis (Sakai et al., 1978; Hajiroussou et al., 1979), bacteremia (Calubiran et al., 1990), septicemia (Casadevall et al., 1992), peritonitis (Glupczynski et al., 1984), and neonatal infections in intensive care units. Their pathogenicity toward animals is not known. Induction of monokine production in human mononuclear cells (Flad and Ulmer, 1995) and stimulation of phagocytosis and

phagosome-lysosome fusion in human neutrophils (Miyazaki et al., 1995) have been reported.

DIFFERENTIATION OF THE GENUS *SPHINGOMONAS* FROM OTHER GENERA

Together with the results of phylogenetic analysis of 16S rDNA nucleotide sequence, the presence of glucuronosyl ceramide is the key characteristic that defines the genus *Sphingomonas* and the family *Sphingomonadaceae* (Figs. BXII.α.85, BXII.α.86, BXII.α.87, and BXII.α.88). Thus it is not enough to refer to the presence of sphingoglycolipid (correctly glucuronosyl ceramide) as “sphingolipid” or even “long chain bases” to define an organism as a member of the genus *Sphingomonas*. The presence of 2-OH myristic acid as the major hydroxy fatty acid and the absence of any kind of 3-hydroxy acid are also important characteristics throughout the family *Sphingomonadaceae*, however, nonpolar myristic acids are present instead of a 2-OH myristic acid in the facultative anaerobic species *Zymomonas mobilis* (Fig. BXII.α.86).

TABLE BXII.α.84. (cont.)

	Species and Strains																			
Fatty acids, % of total	<i>Sphingomonas roseiflava</i> EY 4345 ^T	<i>Sphingomonas sanguinis</i> EY 2397 ^T	<i>Sphingomonas sfygia</i> EY 4297 ^T	<i>Sphingomonas subarctica</i> EY 4251 ^T	<i>Sphingomonas suberifaciens</i> EY 4204 ^T	<i>Sphingomonas subterranea</i> EY 4298 ^T	<i>Sphingomonas taejonensis</i> EY 4377 ^T	<i>Sphingomonas terrae</i> EY 4207 ^T	<i>Sphingomonas trueperi</i> EY 4218 ^T	<i>Sphingomonas ursincola</i> EY 4250 ^T	<i>Sphingomonas wittichii</i> EY 4224 ^T	<i>Sphingomonas xenophaga</i> EY 4343 ^T	<i>Sphingomonas yanoikuyae</i> EY 4208 ^T	<i>Erythrobacter longus</i> EY 4203 ^T	<i>Erythrobacter litoralis</i> EY 4222 ^T	<i>Erythromicrobium ramosum</i> EY 4223 ^T	<i>Porphyrobacter tepidarius</i> EY 4231 ^T	<i>Porphyrobacter neustonensis</i> EY 4230 ^T	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i> EY 4209 ^T	<i>Zymomonas mobilis</i> subsp. <i>pomaceae</i> EY 4210 ^T
C _{14:1}																			tr	tr
C _{14:0}	tr	2	1		2	tr	tr		tr		tr	tr	tr						8	6
C _{15:0 iso}																				
C _{15:0}			tr		tr	tr	6	2						tr					tr	
C _{16:1 ω7c}	13	3	18	6	13	11	3	4	1	9	6	22	14	tr	2	2			tr	
C _{16:1}	tr	tr	tr	2	1	tr	tr	tr		2	2	2	2				tr		2	tr
C _{16:0}	10	12	3	8	4	7	4	2	8	5	12	7	9	4	tr	11	2	5	6	7
C _{17:1}			1		tr	2	7	8						4						
C _{17:0 cyclo}	3	tr	4		6	11	45	45		10	tr	tr	tr	2	1	1	tr	7		
C _{17:0}		tr	tr		1	2	6	4		10			tr	3	6	2	tr			
C _{18:2}														19						
C _{18:1 ω9t}	57	51	49	57	51	52	6	13	77	50	54	57	52	50	73	68	73	58	80	68
C _{18:1 ω7c}																				
C _{18:1}		1	1	tr		tr		tr		1	tr	1	2		tr	tr	2	2		
C _{18:0}		2	1	tr	tr	tr	tr	tr	tr	tr		tr	tr	tr		tr	1	1		
C _{19:1}								tr						1						tr
C _{19:0 cyclo}					tr			1			3									tr
C _{19:0}																tr				
C _{14:0 2OH}	16	28	18	17	13	10	1	2	13	9	10	10	14	6	7	11	16	8		
C _{15:0 2OH}			3		2	3	17	13						7			tr			
C _{16:1 2OH}					tr					tr				2	5	1				
C _{16:0 2OH}				7	tr			tr		2			2	tr	3	2	3	4		
C _{14:0 3OH}																				
C _{16:0 3OH}																				
Other				2	3		4	2		tr	11		3		2	tr	tr	14	3	16
Total	99	99	99	99	96	98	99	96	99	98	98	99	98	98	99	98	97	99	99	97

^aHydrolysis was carried out in HCl-methanol(1:5,v/v), 100°C, 3 h.^bAbbreviations: tr, <1.0%; cyclo, cyclopropanoic acid; nd, not determined.^cData from Lee et al. (2001).

TAXONOMIC COMMENTS

The genus *Sphingomonas** was so named by Yabuuchi et al. (1990a, b) because of the presence of a specific sphingoglycolipid (SGL-1) containing glucuronic acid as the carbohydrate moiety of its molecule (Yamamoto et al., 1978). SGL-1 was first found in the cellular lipids of the type strain of *Flavobacterium devorans* ATCC 10829. The species *Flavobacterium devorans* (Zimmermann 1890) Bergey et al. 1923b was cited in the Approved Lists of Bacterial Names (Skerman et al., 1980) and maintained its nomenclatural standing. However, ATCC strain 10829 was the only strain preserved in a culture collection and no other living cultures of the

species were available. Furthermore, the history of the species revealed that ATCC 10829 was a misidentified later isolate and not the holotype strain of the species (Yabuuchi et al., 1979). Thus, when the strain was later classified as *Pseudomonas paucimobilis*, the priority of specific epithet *devorans* over *paucimobilis* could not be accepted (Yabuuchi et al., 1979). Owen and Jackman (1982) revealed the close relationship between the type strains of *Pseudomonas paucimobilis* and *F. devorans* by a high DNA-DNA hybridization ratio (93 ± 6%) and protein pattern similarity (87%).

Based on the results of a phylogenetic analysis of 16S rDNA and the presence of sphingoglycolipid in cellular lipids of the type strains, the following genera have been placed into the genus *Sphingomonas* by Yabuuchi et al. (1999b): "*Rhizomonas*" van Bruggen et al. 1990, 186; *Blastomonas* Sly and Cahill 1997, 567 emend. Hiraishi et al. 2000a; and *Erythromonas* Yurkov et al. 1997, 1177.

The 16S rDNA sequence similarity of type strains of 34 *Sphin-*

*Editorial Note: Readers should be advised that there are conflicting opinions regarding the taxonomic status of the genus *Sphingomonas*. At this time, there is no strong phenotypic support for the genera *Novosphingobium*, *Sphingobium*, and *Sphingopyxis*, which are well defined in phylogenetic models based on 16S rDNA sequences. Therefore, Yabuuchi et al. (2002) regard these genera as junior objective synonyms of *Sphingomonas*.

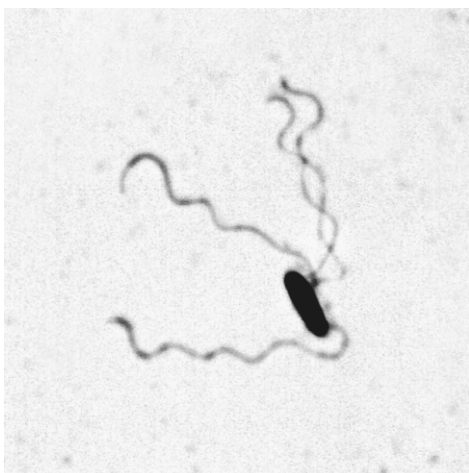


FIGURE BXII.α.90. Flagellar morphology of *Sphingomonas trueperi* EY 4218^T. Four flagella around the soma. Leifson flagella stain.

gomonas species to the type strain of *S. paucimobilis*—the type species of the genus—ranges from 98.1% to 91.0% (see Table BXII.α.80 in the description of the order *Sphingomonadales*). Because of the rapid increase in the number of species, the diversity of metabolic ability, and the wide distribution of organisms in various ecological niches, the definition of the genus *Sphingomonas* has been subjected to much taxonomic discussion chiefly based on the results of phylogenetic analysis of 16S rDNA nucleotide sequences (Takeuchi et al., 1994, 2001; Balkwill et al., 1997; Kämpfer et al., 1997; Hiraishi et al., 2000a; Stolz et al., 2000). Although there has been a proposal to split the genus *Sphingomonas* into four genera, *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (Takeuchi et al., 2001), on the basis of phylogenetic analysis of 16S rDNA sequences and polyamine profiles, from the practical aspect, it is essential that any genus not be delineated by phylogenetic analysis alone, but also

be distinguished by a combination of phenotypic tests, which are easily performed in every microbiological laboratory. The phylogenetic tree derived from 16S rDNA sequence analysis seems likely to support splitting the genus, but an examination of the phenotypic characteristics of 27 type strains among the 33 species currently assigned to the genus *Sphingomonas* in relation to their phylogeny indicates that—as shown in Fig. BXII.α.95—there is no phenotypic evidence to support this proposal (Yabuuchi et al., 2002). We therefore concluded that *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* are junior objective synonyms of *Sphingomonas*, and it is unreasonable to exclude *S. yanoikuyae*, *S. herbicidovorans*, *S. chlorophenolica*, *S. rosa*, *S. subarctica*, *S. stygia*, *S. subterranea*, *S. aromaticivorans*, *S. capsulata*, *S. macrogoltabidus*, and *S. terrae* from the genus *Sphingomonas*. A dendrogram showing the relationships among the 16S rDNA sequences of *Sphingomonas* species is given in Fig. BXII.α.96. Furthermore, Takeuchi et al. (2001) utilized the rejected generic name “*Rhizomonas*” in combination with the specific epithet *suberifaciens*. It is an obvious violation of the Code. “*Rhizomonas suberifaciens*” was transferred to the genus *Sphingomonas*, as *Sphingomonas suberifaciens* (Yabuuchi et al., 1999a).

Because of the detection of bacteriochlorophyll *a* and *puf* genes encoding the proteins related to photosynthetic activities from both *Blastomonas natatoria* and *Erythromonas ursincola*, together with phenotypic, chemoanalytic, and phylogenetic data, Hiraishi et al. (2000a) published an emended description of *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997 and a proposal of *Blastomonas ursincola* comb. nov. These two species had been assigned to the genus *Sphingomonas* as *Sphingomonas natatoria* (Sly 1985) Yabuuchi et al. 1999b and *Sphingomonas ursincola* (Yurkov et al. 1997) Yabuuchi et al. 1999b, based on the presence of glucuronosyl seramide (SGL-1) in their cellular lipids and the results of phylogenetic analysis of 16S rDNA sequences. In spite of the report and proposal by Hiraishi et al. (2000a), phototrophic ability is widely distributed among procaryotes, and its presence does not necessarily indicate a close phylogenetic relationship among these taxa (Kondratieva et al., 1992).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *SPHINGOMONAS*

The differential features and other characteristics of the species of *Sphingomonas* are listed in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89. Data on members of the genera *Erythrobacter*, *Porphyrobacter*, *Sandaracinobacter*,

and *Zymomonas* are provided for comparison in Tables BXII.α.84 (fatty acid composition), BXII.α.85 (morphological and biochemical characteristics), and BXII.α.88 (oxidative acid production).

List of species of the genus *Sphingomonas*

1. ***Sphingomonas paucimobilis*** (Holmes, Owen, Evans, Malnick and Wilcox 1977a) Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116) (*Pseudomonas paucimobilis* Holmes, Owen, Evans, Malnick and Wilcox 1977a, 133.) *pau.ci.mo' bi.lis*. L. adj. *paucis* few; L. adj. *mobilis* mobile; M.L. fem. adj. *paucimobilis* intended to mean few motile cells.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Colonies are convex and deep yellow on ordinary peptone media. Soma size is $0.7 \times 1.4 \mu\text{m}$ (Holmes et al., 1977a). A few cells are motile with single polar flagellum. Some cells form a rosette-like arrangement. In addition to SGL-1, SGLs-2, 3, and 4 are detected on TLC

(Table BXII.α.83, Figs. BXII.α.85 and BXII.α.87). SGL induces monokine production in human mononuclear cells (Krziwon et al., 1995) and stimulates phagocytosis and phagosome-lysosome fusion in human neutrophil cells (Miyazaki et al., 1995). Major polyamine is homospermidine (Busse and Auling, 1988; Hamana and Matsuzaki, 1993; Segers et al., 1994; Takeuchi et al., 1995; Takeuchi et al., 2001); one strain was reported to produce trace amounts of spermidine (Segers et al., 1994). The structure and function of sphingan (Pollock, 1993; Lobas et al., 1994; Pollock et al., 1994; Sutherland, 1994) and lyases (Sutherland and Kennedy, 1996) have been reported. Human clinical cases of infection have been reported (Crane et al., 1981; Glupczynski et al., 1984; Calubiran et al., 1990; Casadevall et al., 1992; Decker et al., 1992; Lemaitre et al., 1996). Type strain was isolated from hospital respirators.

TABLE BXII.α.85. Morphological, physiological, and biochemical characteristics of 42 type strains of 40 species and 2 subspecies in *Sphingomonadaceae*^a

Substrate or test	<i>Sphingomonas paucimobilis</i> EY 2395 ^T	<i>Sphingomonas adhaesiva</i> EY 4215 ^T	<i>Sphingomonas alaskensis</i> EY 4374 ^T	<i>Sphingomonas aquatilis</i> KCTC 2881 ^{Tb}	<i>Sphingomonas aromaticivorans</i> EY 4296 ^T	<i>Sphingomonas chlorophenolica</i> EY 4219 ^T	<i>Sphingomonas capsulata</i> EY 4216 ^T	<i>Sphingomonas asaccharolytica</i> EY 4229 ^T	<i>Sphingomonas chungbukensis</i> EY 4375 ^T	<i>Sphingomonas cloacae</i> EY 4361 ^T	<i>Sphingomonas echinoides</i> EY 4340 ^T	<i>Sphingomonas herbicidovorans</i> EY 4344 ^T	<i>Sphingomonas korensis</i> EY 4376 ^T	<i>Sphingomonas macrogoltabidus</i> EY 4304 ^T	<i>Sphingomonas mali</i> EY 4341 ^T	<i>Sphingomonas melonis</i> EY 4350 ^T	<i>Sphingomonas natatoria</i> EY 4220 ^T	<i>Sphingomonas parapaucimobilis</i> EY 4213 ^T	<i>Sphingomonas pituitosa</i> EY 4370 ^T	<i>Sphingomonas pruni</i> EY 4228 ^T	<i>Sphingomonas rosea</i> EY 4227 ^T
Gram-negative rod shaped	+	+	+	+	+	+	+	+	Ov	+	+	+	+	+	+	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in the presence of 3% NaCl	+	+	+	-	-	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+
Alkapton production	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Pigmentation of colonies	DY	DY	Y	Y	DY	LY	DY	LY	Y	GW	LY	Y	Y	GW	LY	Y	O	DY	DY	GW	GW
Bacteriochlorophyll <i>a</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Oxidase	-	-	+	+	+	+	-	+	+	+	+	+	+	-	+	w	-	-	-	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of:																					
Esculin	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	-	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	-	+	+	+	+	-
Tween 80	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	-
Citrate, Simmons	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+	-	-	-
Malonate	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	NG	-	-	-	+
Arginine, Moller	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine, ornithine, Moller	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from nitrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrite from nitrate	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-
Zn test on negative NO ₂ test	+	+	+	-	+	+	+	+	+	+	nd	+	+	+	+	+	+	-	nd	+	+
Phenylalanine deaminase	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acylamidase	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	NG	-
DNase	-	-	-	-	+	NG	-	+	-	-	-	-	-	-	+	-	-	-	-	NG	+

^aSymbols: +, positive reactions; -, negative reactions; Ov, ovoid; Y, yellow; LY, light yellow; GW, grayish white; O, orange; PY, pale yellow; OB, orange brown; B, brown; RO, reddish orange; YO, yellowish orange; NG, no growth; nd, not determined.

^bData from Lee et al. (2001). Blank space, no data available.

^cData from Yurkov et al. (1997). Blank space, no data available.

The mol% G + C of the DNA is: 62–64 (HPLC).

Type strain: ATCC 29837, DSM 1098, EY2395, GIFU 2395, IAM 12576, IFO 11385, JCM 7516, LMG 1227, NCTC 11030.

GenBank accession number (16S rRNA): U37337, X72722.

2. ***Sphingomonas adhaesiva*** Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116.)

ad.hae.si.va. L. adhaerere adhere; *adhaesiva* intended to mean sticking to agar medium.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Cells have polar monotrichous flagella. Circular and yellow-pigmented colonies stick to agar medium, but

corrosion of the agar has not been observed. No growth occurs on Simmons' citrate medium. Acid production in OF medium from carbohydrates takes 4 or more days of incubation. Major long-chain bases are C_{18:0} (39%) and C_{21:1} (26%); major fatty acids are C_{18:1 cis} (46%) and C_{14:0 2OH} (23%). Major polyamine is homospermidine (Hamana and Matsuzaki, 1993; Segers et al., 1994; Takeuchi et al., 1995). The type strain was isolated from ultraviolet-irradiated water at a surgical operation theater.

The mol% G + C of the DNA is: 67.2 (HPLC).

Type strain: ATCC 51229, CCUG 27290, DSM 7418, EY 4215, GIFU 11458, IFO 5099, JCM 7370, LMG 0922.

GenBank accession number (16S rRNA): D16146, X72720.

3. ***Sphingomonas alaskensis*** Vancanneyt, Schut, Snuwaert, Goris, Swings and Gottschal 2001, 78^{VP}

TABLE BXII.α.85. (cont.)

Substrate or test	<i>Sphingomonas roseiflava</i> EY 4345 ^T	<i>Sphingomonas sanguinis</i> EY 2397 ^T	<i>Sphingomonas stygia</i> EY 4297 ^T	<i>Sphingomonas subarctica</i> EY 4251 ^T	<i>Sphingomonas suberifaciens</i> EY 4204 ^T	<i>Sphingomonas subterranea</i> EY 4298 ^T	<i>Sphingomonas taejonensis</i> EY 7377 ^T	<i>Sphingomonas terrae</i> EY 4207 ^T	<i>Sphingomonas trueperi</i> EY 4218 ^T	<i>Sphingomonas ursincola</i> EY 4250 ^T	<i>Sphingomonas vititichii</i> EY 4224 ^T	<i>Sphingomonas xenophaga</i> EY 4343 ^T	<i>Sphingomonas yanoikuyae</i> EY 4208 ^T	<i>Erythrobacter longus</i> EY 4203 ^T	<i>Erythrobacter litoralis</i> EY 4222 ^T	<i>Erythromicrobium ramosum</i> EY 4223 ^T	<i>Porphyrobacter neustonensis</i> EY 4230 ^T	<i>Porphyrobacter tepidarius</i> EY 4231 ^T	<i>Sandaracinobacter sibiricus</i> RB 16-17 ^{Tc}	<i>Z. mobilis</i> subsp. <i>mobilis</i> EY 4209 ^T	<i>Z. mobilis</i> subsp. <i>pomacii</i> EY 4209 ^T
Gram-negative rod shaped	+	+	+	+	+	+	+	+	+	Ov	+	+	+	+	+	+	+	Ov	+	+	+
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in the presence of 3% NaCl	-	+	-	+	-	-	-	+	+	-	+	+	-	+	+	+	-	-	+	+	+
Alkapton production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pigmentation of colonies	O	DY	LY	LY	Y	DY	PY	DY	LY	OB	GW	DY	GW	B	RO	O	RO	O	YO	GW	GW
Bacteriochlorophyll <i>a</i>	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-
Oxidase	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+
Fermentation of glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Hydrolysis of:																					
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	nd	nd
Gelatin	+	+	+	+	-	+	-	+	-	-	+	-	-	+	-	-	-	+	-	-	-
Starch	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-
Tween 80	+	+	-	-	-	-	+	-	-	-	+	-	-	w	+	+	+	+	-	-	-
Citrate, Simmons	-	-	-	+	-	-	-	-	-	-	+	-	+	nd	nd	+	-	+	-	-	-
Malonate	-	-	-	-	-	+	-	-	-	-	-	-	-	nd	nd	nd	-	-	-	nd	nd
Arginine, Moller	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	-	-	-	-	-
Lysine, ornithine, Moller	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	-	+	-	nd	nd
Gas from nitrate	+	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	+	-	-	-	-	-
Nitrite from nitrate	+	-	-	-	-	-	-	-	-	+	+	-	-	nd	nd	+	-	+	-	-	-
Zn test on negative NO ₂ test	-	+	+	+	+	+	+	+	+	+	+	+	+	nd	nd	nd	+	+	-	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	+	-	-	nd	nd	-	-	+	-	nd	nd
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	nd	nd
Acylamidase	-	-	-	+	-	-	-	-	-	-	+	-	-	nd	nd	nd	-	-	-	nd	nd
DNase	-	-	+	+	-	+	-	+	-	-	-	-	-	nd	nd	+	NG	-	-	nd	nd

^aSymbols: +, positive reactions; -, negative reactions; Ov, ovoid; Y, yellow; LY, light yellow; GW, grayish white; O, orange; PY, pale yellow; OB, orange brown; B, brown; RO, reddish orange; YO, yellowish orange; NG, no growth; nd, not determined.

^bData from Lee et al. (2001). Blank space, no data available.

^cData from Yurkov et al. (1997). Blank space, no data available.

a.las.ken'sis. M.L. adj. *alaskensis* pertaining to Alaska, the source of the type strain.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Motile small rod. Soma size 0.2–0.5 × 0.5–3.0 µm. Although isolated at low temperatures (4–8°C), the optimal growth temperature for the type strain is approximately 37°C. Growth occurs at 44–48°C. Does not grow in Bacto-OF medium. Acid is produced in Bacto-CTA medium from maltose and trehalose, but not from D-glucose (Table BXII.α.88). For results with the API 20NE and API 50CH systems, see Vancanneyt et al. (2001). Isolated from seawater from Alaska.

The mol% G + C of the DNA is: 65 (HPLC).

Type strain: DSM 13593, EY 4374, LMG 18877, RB 2256.

GenBank accession number (16S rRNA): AF145754.

4. *Sphingomonas aquatilis* Lee, Shin, Yoon, Takeuchi, Pyun and Park 2001, 1495^{VP}

a.qua.til'is. L. fem. adj. *aquatilis* aquatic, growing in water.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.88, and BXII.α.89, with the following additional information. Motile by a single polar flagellum. Colonies are circular, entire, low convex, smooth, opaque and yellow. Gelatin is not liquefied. Esculin is not hydrolyzed. D-Melibiose, D-sucrose, glucose, malate, maltose, N-acetylglucosamine, rhamnose, and salicin are assimilated, but 2-ketogluconate, 5-ketogluconate, adipate, caprate, citrate, D-ribose, D-sorbitol, gluconate, glycogen, L-arabinose, mannose, and phenylacetate are not assimilated. Acid is weakly produced oxidatively from glucose and rhamnose, but not from glycerol. Isolated from natural mineral water in Taejeon City, Korea.

The mol% G + C of the DNA is: 63 (HPLC).

Type strain: JSS7, KCTC 2881, KCCM 41067.

GenBank accession number (16S rRNA): AF131295.

Additional Remarks: Reference strain: EY 4383, JSS28, KCTC 2883, KCCM 41066.

5. *Sphingomonas aromaticivorans* Balkwill, Drake, Reeves,

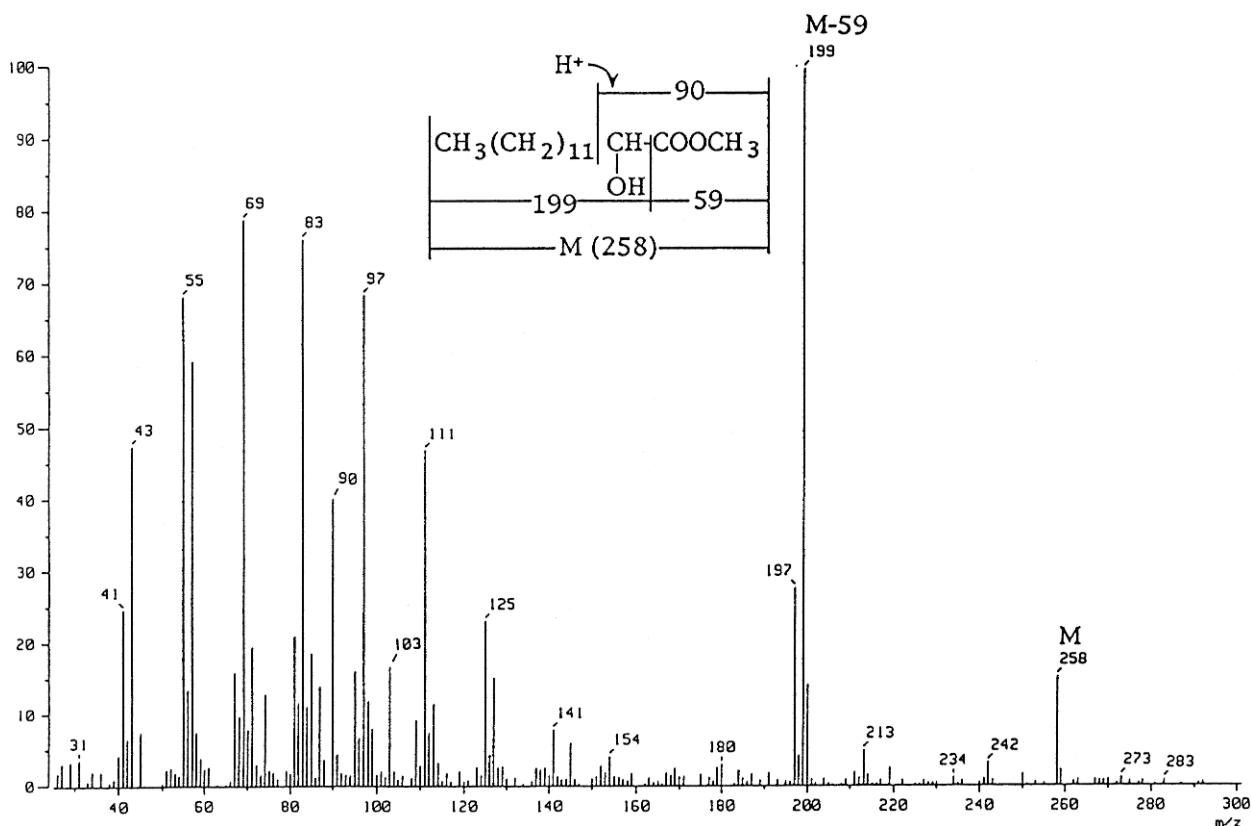


FIGURE BXII.α.91. Mass spectrum of the reaction product of H_2O_2 -dependent α -hydroxylation of myristic acid. The mass spectrum of the methylated product gave ions of m/z 258 and 199, which were identical to those of methyl-2-hydroxymyristic acid (*M*) and its characteristic fragment (*M*-59), respectively. (Reproduced with permission from I. Matsunaga et al., Federation of European Biochemical Societies Letters, 386: 252–254, 1996, ©Elsevier Science B.V., Amsterdam.)

Fredrickson, White, Ringelberg, Chandler, Romine, Kennedy and Spadoni 1997, 199^{VP}*
a.ro.ma.ti.ci'vo.rans. N.L. n. *aromaticus* aromatic compound; L. part. *vorans* eating; *aromaticivorans* eating aromatic compounds.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Nonmotile. Colonies are circular entire low convex smooth opaque and deep yellow. SGL-1 is present but not SGL-1'. In addition to $\text{C}_{14:0} 2\text{OH}$, $\text{C}_{15:0} 2\text{OH}$ is detected as a minor component. Major polyamine is spermidine (Busse et al., 1999; Takeuchi et al., 2001). Isolated from saturated Atlantic coastal plain terrestrial subsurface sediments.

The mol% *G* + *C* of the DNA is: 62.9–65.0 (HPLC).

Type strain: F199, DSM 12444, EY 4296, JCM 16084, SMCC F199.

GenBank accession number (16S rRNA): U20756.

Additional Remarks: Reference strains include strains B0522 (SMCC B0522) and B0695 (SMCC B0695).

6. *Sphingomonas asaccharolytica* Takeuchi, Sakane, Yanagi, Yamasato, Hamana and Yokota 1995, 341^{VP}

a.sac.cha.ro.ly'ti.ca. Gr. pref. *a* not; Gr. n. *sacchar* sugar; Gr. adj. *lytica* able to loosen; M.L. fem. adj. *asaccharolytica* not digesting sugar.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Nonmotile because neither actively motile in wet mount preparation nor demonstrates any spreading growth on semisolid motility agar plate. Colonies are light yellow. In spite of the specific epithet, acid is produced oxidatively in OF basal medium from eight kinds of carbohydrates, including glucose and maltose (Table BXII.α.88). β -Galactosidase positive. 3-Ketolactose is produced and the major polyamine is homospermidine (Takeuchi et al., 1995; Takeuchi et al., 2001); Kämpfer et al. (1997) reported the production of minor amounts of spermidine in addition to homospermidine. Isolated from the roots of *Malus* spp. (apple) in Tsukuba City, Japan.

The mol% *G* + *C* of the DNA is: 64.8 (HPLC).

Type strain: Y-345, ATCC 51839, DSM 10564, EY 4229, IFO 15499, JCM 10279, LMG 17539.

GenBank accession number (16S rRNA): Y09639.

7. *Sphingomonas capsulata* (Leifson 1962) Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto,

*Editorial Note: *Novosphingobium aromaticivorans* (Balkwill et al. 1997) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. aromaticivorans* (Yabuuchi et al., 2002).

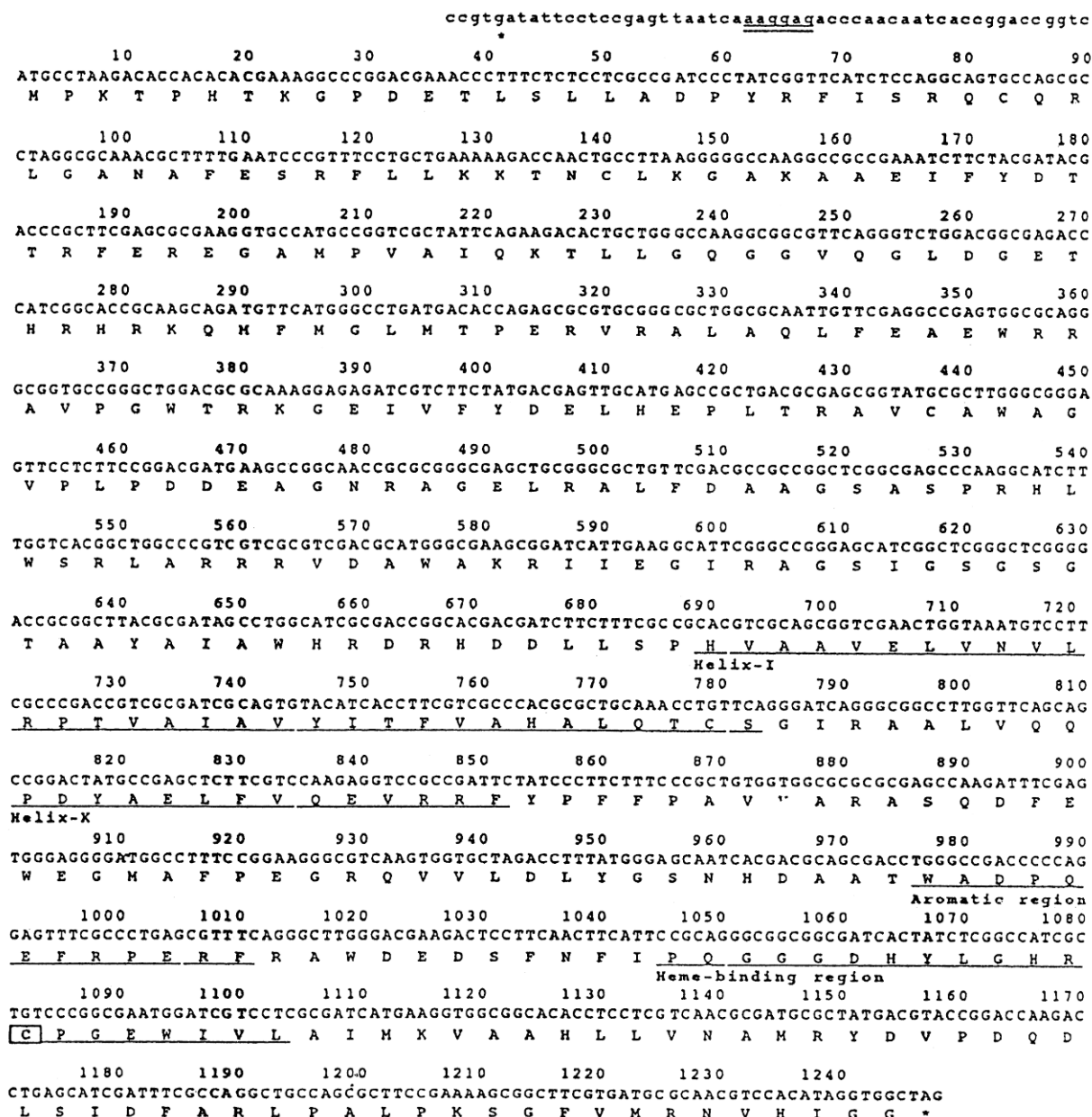


FIGURE BXII.α.92. Nucleotide sequence of 1.3-kb fragment containing the fatty acid α-hydroxylase gene and deduced amino acid sequence. The deduced amino acid sequence is represented under the nucleotide sequence by single-letter code. The asterisk and double underline indicate the termination codon and putative Shine-Dalgarno sequence, respectively. Helix-I, helix-K, aromatic region, and heme-binding region are underlined. Heme-binding cysteine is boxed. (Reproduced from I. Matsunaga et al., Journal of Biological Chemistry, 272: 23592–23596, 1997, ©American Society for Biochemistry and Molecular Biology, Inc.)

Ezaki and Yamamoto 1990a, 117) (*Flavobacterium capsulatum* Leifson 1962, 161.)*
cap.su.la' ta. L. n. *capsula* a small chest, capsule; M.L. fem. adj. *capsulata* encapsulated.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Nonmotile without flagellum. Produces defi-

nately yellow colonies. In spite of the specific epithet (Leifson, 1962), the organism lost its ability to produce capsule. Phenylalanine deaminase positive. Major polyamine reported as spermidine (Busse and Auling, 1988; Hamana and Matsuzaki, 1993; Segers et al., 1994; Takeuchi et al., 1995; Takeuchi et al., 2001). Isolated from distilled water.

The mol% G + C of the DNA is: 63.1 (HPLC).

Type strain: ATCC 14666, DSM 30916, EY 4216, GIFU 11526, IFO 12533, JCM 7508, LMG 2830, NCIMB 9890.

GenBank accession number (16S rRNA): D16147.

8. *Sphingomonas chlorophenolica* Nohynek, Suhonen, Nurmiäho-Lassila, Hantula and Salkinoja-Salonen 1996b, 625^{VP}

*Editorial Note: *Novosphingobium capsulatum* (Leifson 1962) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. capsulata* (Yabuuchi et al., 2002).

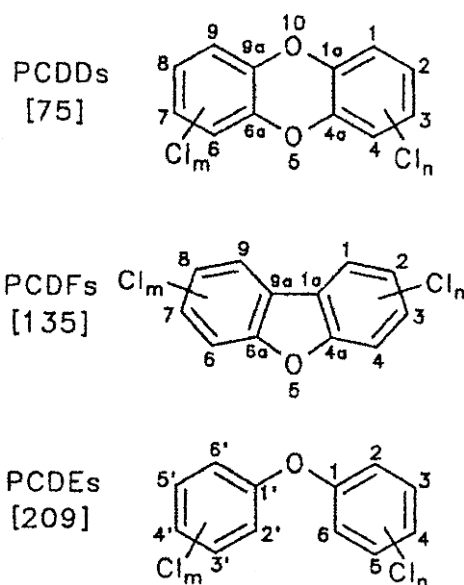


FIGURE BXII.α.93. Structure of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and diphenyl ethers (PCDEs). The number of possible congeners is given in brackets. (Reproduced from R.-M. Wittich. 1998. In Wittich (Editor) *Biodegradation of Dioxins and Furans*. Springer-Verlag, Berlin, and R.G. Landes Co., pp. 1–28.)

(Effective publication: Nohynek, Suhonen, Nurmiaho-Lasila, Hantula and Salkinoja 1995, 536.)*

chlor.o.phen.o' li.ca. L. fem. adj. *chloro* containing chlorine; N.L. n. *pheno* phenol; L. fem. adj. *chlorophenolica* relating to chlorophenols.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Rods vary in size, 0.3–0.7 × 1.0–3.5 μm. Acylamidase and DNase are positive, but hydrolysis of starch and Tween 80 are negative. Fimbriae or filaments may occur. Major polyamine is spermidine (Busse et al., 1999; Takeuchi et al., 2001).

Isolated from soil contaminated with wood preserving chlorophenols.

The mol% G + C of the DNA is: 63–67 (*T_m*).

Type strain: ATCC 33790, DSM 6284, EY 4219, IFO 16172, JCM 10275, LMG 17554.

GenBank accession number (16S rRNA): U60171, X87161.

9. ***Sphingomonas chungbukensis*** Kim, Chun, Bae and Kim 2000a, 1646^{VP}
chung.bu.ken'sis. M.L. adj. *chungbukensis* pertaining to Chungbuk National University.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Curved rods, 0.5–1 × 0.5–3.0 μm. Nonmotile. Colonies are smooth and yellow. Produce a great quantity of extracellular polysaccharide. Esculin and Tween 80 are not hydrolyzed. The major respiratory quinone is ubiqui-

none 10. Isolated from chemically contaminated freshwater sediment in Taejeon, Republic of Korea.

The mol% G + C of the DNA is: 63 (*T_m*).

Type strain: DJ77, EY 4375, IMSNU 11152, JCM 11454, KCTC 2955.

GenBank accession number (16S rRNA): AF159257.

10. ***Sphingomonas cloacae*** Fujii, Urano, Ushio, Satomi and Kimura 2001, 608^{VP}
clo.a' cae. L. n. *cloaca* sewer, the source of the organism.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Cells are 1.1–1.4 × 2.0–3.1 μm. Motile. Colonies are opaque and white. Unable to grow at 4° or 42°C. Both SGL-1 and SGL-1' are present. The major isoprenoid quinone is ubiquinone Q-10. Isolated from wastewater of a sewage-treatment plant in Tokyo.

The mol% G + C of the DNA is: 63 (HPLC).

Type strain: S-3, EY 4361, IAM 14885, JCM 10874.

GenBank accession number (16S rRNA): AB040739.

11. ***Sphingomonas echinoides*** (Heumann 1962) Denner, Kämpfer, Busse, and Moore 1999, 1108^{VP} (*Pseudomonas echinoides* Heumann 1962.)
e.chi.noi'des. Gr. adj. *echinos* spiny appearance; Gr. n. *eidus* form, shape; M.L. adj. *echinoides* spiny shaped.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Curved rods 0.8 × 1.9 μm, with sharp ends. Motile by polar flagella of 1.9 μm wavelength. Colonies are light yellow. Form cell aggregates (rosettes) both on solid and in liquid media. β-Galactosidase positive. For chromatogram of lipids, see Fig. BXII.α.85. Major polyamine is homospermidine (Denner et al., 1999; Takeuchi et al., 2001). Denner et al. (1999) also reported production of spermidine. Isolated as a laboratory contaminant on a nutrient agar plate.

The mol% G + C of the DNA is: 65.8 (*T_m*) (Owen and Jackman, 1982).

Type strain: ATCC 14820, DSM 1805, EY 4340, ICBP 2835, IFO 15742, JCM 10637, NCIMB 9420.

GenBank accession number (16S rRNA): AB021370, AJ012461.

12. ***Sphingomonas herbicidovorans*** Zipper, Nickel, Angst, and Kohler 1997, 601^{VP} (Effective publication: Zipper, Nickel, Angst and Kohler 1996, 4319.)*
herb.i.ci.do'vo.rans. L. n. *herba* an herb; *cido-cide* joint word to mean killing; L. v. *voro* to devour; M.L. fem. adj. *herbicidovorans* herbicide-devouring, referring to its ability to utilize herbicides as a sole source of carbon and energy.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. The type strain, strain MH (previously designated *Flavobacterium* sp. strain MH) was able to utilize the chiral herbicide (RS)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) as the sole carbon end en-

*Editorial Note: *Sphingobium chlorophenolicum* (Nohynek et al. 1996a) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. chlorophenolica* (Yabuuchi et al., 2002).

**Editorial Note: (Zipper et al. 1997) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. herbicidovorans* (Yabuuchi et al., 2002).

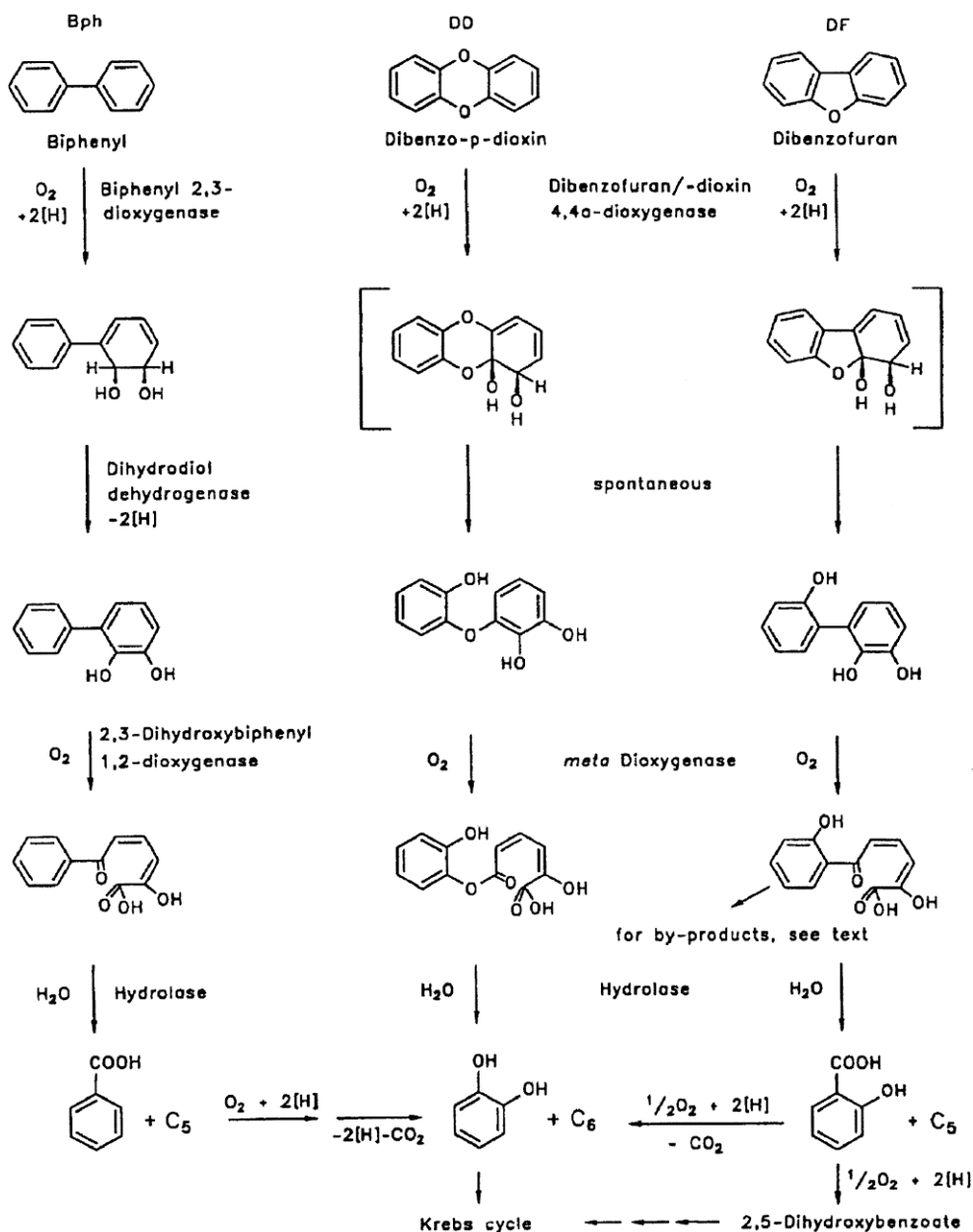


FIGURE BXII.α.94. Pathways for the complete aerobic degradation of biphenyl, dibenzo-*p*-dioxin, and dibenzofuran. (Reproduced from R.-M. Wittich, 1998. In Wittich (Editor) *Biodegradation of Dioxins and Furans*. Springer-Verlag, Berlin, and R.G. Landes Co., pp. 1-28.)

ergy source. Colonies are yellow. Produce the water-soluble brown pigment alkaptone due to accumulation of homogentisic acid by metabolic failure of phenylalanine or tyrosine. A chromatogram of SGL-1 is shown in Fig. BXII.α.85. Major polyamine is spermidine (Busse et al., 1999; Takeuchi et al., 2001). Isolated from soil.

The mol% G + C of the DNA is: 62.5 (HPLC).

Type strain: MH, ATCC 700291, CIP 106705, DSM 11019, EY 4344, LMG 18315.

GenBank accession number (16S rRNA): AB042233.

13. **Sphingomonas koreensis** Lee, Kook Shin, Yoon, Takeuchi, Pyun and Park 2001, 1496^{VP}
ko.re.en'sis. N.L. fem. adj. *koreensis* pertaining to Korea, where the new organisms were isolated.

The characteristics are as described for the genus and

in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Motile rods with a single polar flagellum. Colonies are yellow. Acid is produced oxidatively in OF medium from 6 of 26 carbohydrates. The major isoprenoid quinone is ubiquinone Q-10. Isolated from natural mineral water in Taejon City, Korea.

The mol% G + C of the DNA is: 66 (HPLC).

Type strain: JSS26, EY 4376, JCM 11456, KCTC 2882, KCCM 41069.

GenBank accession number (16S rRNA): AF131296.

14. **Sphingomonas macrogoltabidus** Takeuchi, Kawai, Shimada, and Yokota 1993b, 864^{VP} (Effective publication: Takeuchi, Kawai, Shimada and Yokota 1993a, 236.)*

TABLE BXII.α.86. Selected results of assimilation tests (Biotype 100) by 31 *Sphingomonas* species^{a,b}

Biotype 100, Substrates or tests	<i>S. paucimobilis</i> EY 2395	<i>S. adhaesiva</i> EY 4215	<i>S. alaskensis</i> EY 4374	<i>S. aromaticivorans</i> EY 4296	<i>S. asaccharolytica</i> EY 4229	<i>S. capsulata</i> EY 4216	<i>S. chlorophenolica</i> EY 4219	<i>S. chungbukensis</i> EY 4375	<i>S. cloacae</i> EY 4361	<i>S. echinoides</i> EY 4340	<i>S. herbicidovorans</i> EY 4344	<i>S. korrensis</i> EY 4376	<i>S. macrogoltabidus</i> EY 4304	<i>S. mali</i> EY 4341	<i>S. natatoria</i> EY 4220	<i>S. parapaucimobilis</i> EY 4213
Betaine	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
α-Ketoglutarate	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D(+)Trehalose	+	+	+	—	+	+	—	—	—	+	—	+	+	+	—	+
D-Galacturonate	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
D-Saccharate	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
(-)-Quinate	—	—	—	+	—	+	—	—	—	—	—	—	—	—	—	—
D(-)Ribose	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
<i>m</i> -Hydroxybenzoate	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
<i>trans</i> -Aconitate	+	—	—	—	—	—	—	—	+	—	—	—	—	—	—	+
L(-)-Malate	+	+	—	—	+	+	+	—	—	+	+	+	—	—	—	+
α-L-Rhamnose	—	—	—	+	+	+	—	—	—	—	+	—	—	+	—	+
L-Serine	+	—	—	—	+	—	—	—	—	—	—	+	—	—	—	—
D(-)-Tartrate	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
α-D(+)-Melibiose	+	+	—	+	+	—	—	—	—	—	—	—	—	+	—	+
Protocatechuate	+	—	—	—	—	+	+	+	—	—	—	—	+	—	+	—
5-Keto-L-Gluconate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
<i>meso</i> -Tartrate, D(+)-malate, caprylate,	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	+
D(+)-Galactose	+	+	—	—	+	+	—	+	—	—	—	—	—	+	—	+
L-Tryptophan	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>cis</i> -Aconitate	+	—	—	—	+	—	—	—	+	—	—	—	—	—	—	+
D-Glucosamine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D-Lyxose, <i>i</i> -erythritol, 2-keto-D-gluconate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
d-Alanine	—	—	—	—	+	—	—	+	+	—	—	—	—	—	—	—
Phenylacetate	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
L-Aspartate	+	+	—	—	+	+	+	—	—	—	—	+	+	+	—	—
Succinate	+	+	—	—	+	+	+	—	—	—	+	+	—	—	—	+
1-O-Methyl-α-galactopyranoside	+	—	—	+	—	—	—	—	—	—	—	—	—	—	—	+
D-Tagatose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D,L-Glycerate	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
<i>p</i> -Hydroxybenzoate	+	—	—	+	—	+	+	+	+	—	—	—	—	—	—	—
Mucate	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—

^aFor symbols see standard definitions.^bThe 17 substrates not assimilated by the 31 strains are dulcitol, L(+)-sorbitol, D(+)-arabitol, L(-)-arabitol, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, L(+)-tartrate, 3-phenylpropionate, *m*-coumarate, trigonelline, histamine, histidine, D,L-α-amino-*n*-valerate, ethanolamine, malonate, and 3-O-methyl-D-glucopyranose.

mac.ro.gol.ta' bi.dus. M.L. fem. n. *macrogol* a trade name for a polyethylene glycol product; L. adj. *tabidus* dissolving; M.L. fem. adj. *macrogoltabida* polyethylene glycol dissolving.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Motile by a single polar flagellum. Colonies are grayish white. Polyethylene glycol 4000 is assimilated. Major polyamine is spermidine (Takeuchi et al., 1995; Takeuchi et al., 2001). Isolated from soil.

The mol% G + C of the DNA is: 63.2–65.0 (HPLC).

Type strain: CIP 104196, DSM 8826, EY 4304, IFO 15033, JCM 10192, LMG 17324.

GenBank accession number (16S rRNA): D13723.

15. ***Sphingomonas mali*** Takeuchi, Sakane, Yanagi, Yamasato, Hamana and Yokota 1995, 341^{VP}

mal' i. M.L. gen. n. *mali* of *Malus*, the apple genus, the source of the organism.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Cells are motile. Colonies are light yellow. DNase positive, but does not hydrolyze gelatin and starch. Major polyamine is homospermidine (Takeuchi et al., 1995; Takeuchi et al., 2001). Isolated from the roots of *Malus* spp. (apple) in Tsukuba City, Japan.

The mol% G + C of the DNA is: 65.4–65.9 (HPLC).

Type strain: Y-547, ATCC 51480, DSM 10565, EY 4341, IFO 15500, JCM 10193, LMG 17331.

GenBank accession number (16S rRNA): Y09638.

16. ***Sphingomonas melonis*** Buonauro, Stravato, Kosako, Fujiwara, Naka, Kobayashi, Cappelli and Yabuuchi 2002, 2086. *me.lo' nis*. M.L. n. *melo* melon; L. gen. n. *melonis* of melon (*Cucumis melo* var. *inodorus*, Spanish melon), referring to the fruit of the plant for which the organism was pathogenic.

*Editorial Note: *Sphingopyxis macrogoltabida* (Takeuchi et al. 1993a) Takeuchi et al. 2001, 1416 is a junior objective synonym of *S. macrogoltabidus* (Yabuuchi et al., 2002).

TABLE BXII.α.86. (cont.)

Biotype 100, Substrates or tests	<i>S. pituitosa</i> EY 4370	<i>S. pruni</i> EY 4228	<i>S. rosa</i> EY 4227	<i>S. roseiflava</i> EY 4345	<i>S. sanguinis</i> EY 2397	<i>S. stygia</i> EY 4297	<i>S. subarctica</i> EY 4251	<i>S. subterranea</i> EY 4298	<i>S. taenionensis</i> EY 4377	<i>S. terrae</i> EY 4207	<i>S. trueperi</i> EY 4218	<i>S. ursincola</i> EY 4250	<i>S. wittichii</i> EY 4924	<i>S. xenophaga</i> EY 4343	<i>S. yanoikuyae</i> EY 4208	No. of positive EY strains	No. of negative EY strains
Betaine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	30
α-Ketoglutarate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	29
D(+) Trehalose	+	+	+	-	+	-	+	-	-	+	+	-	+	-	+	19	12
D-Galacturonate	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	3	28
D-Saccharate	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	3	28
(-)-Quinate	+	-	+	-	-	-	-	+	-	-	-	-	+	-	+	7	24
D(-) Ribose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	2	29
m-Hydroxybenzoate	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	2	29
trans-Aconitate	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	6	25
L(-)-Malate	+	-	-	+	-	-	+	-	-	+	+	-	+	+	+	17	14
α-L-Rhamnose	-	+	+	-	-	-	+	+	-	-	+	-	-	-	+	12	19
L-Serine	-	-	-	-	+	-	+	-	-	-	+	-	+	-	-	7	24
D(-)-Tartarate	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	2	29
α-D(+)-Melibiose	+	+	-	-	+	-	-	-	-	-	+	+	+	-	+	13	18
Protocatechuate	+	-	+	-	-	-	+	+	-	-	+	-	-	+	+	13	18
5-Keto-L-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	30
meso-Tartrate, D(+)-malate, caprylate,	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	29
D(+)-Galactose	+	+	+	-	+	-	+	+	-	-	+	-	+	+	+	17	14
L-Tryptophan	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	1	30
cis-Aconitate	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	7	24
D-Glucosamine	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	2	29
D-Lyxose, D-erythritol, 2-keto-D-gluconate	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1	30
D-Alanine	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	5	26
Phenylacetate	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	3	28
L-Aspartate	+	-	-	-	+	-	+	-	+	-	-	-	+	-	+	14	17
Succinate	+	-	-	+	+	-	+	-	-	+	+	-	+	+	+	17	14
1-O-Methyl-α-galactopyranoside	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	5	26
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2	29
DL-Glycerate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	2	29
p-Hydroxybenzoate	+	-	+	-	-	-	+	+	-	-	-	-	-	+	+	11	20
Mucate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	2	29

^aFor symbols see standard definitions.

^bThe 17 substrates not assimilated by the 31 strains are dulcitol, L(+)-sorbitol, D(+)-arabitol, L(-)-arabitol, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, L(+)-tartrate, 3-phenylpropionate, m-coumarate, trigonelline, histamine, histidine, DL-α-amino-n-valerate, ethanolamine, malonate, and 3-O-methyl-D-glucopyranose.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Non-motile. Colonies are deep yellow. Isolated from fruits of yellow Spanish melons (*Cucumis melo* var. *inodorus*) in Almeria (Spain). Causal agent of brown spot of melon fruits. Organisms identified as strains of *Sphingomonas* sp., had previously been reported as a causative agent of melon fruit disease (Buonaurio et al., 2001).

The mol% G + C of the DNA is: 65 (HPLC).

Type strain: DAPP-PG 224, EY 4350, LMG 19484.

GenBank accession number (16S rRNA): AB055863.

17. ***Sphingomonas natatoria*** (Sly 1985) Yabuuchi, Kosako, Naka, Suzuki and Yano 1999b, 935^{VP} (Effective publication: Yabuuchi, Kosako, Naka, Suzuki and Yano 1999a, 347) (*Blastobacter natatorius* Sly 1985, 40; *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997, 568.)*

na.ta.to' ri.a. M.L. fem. adj. *natatoria* of a swimming place (pool).

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Straight rod. Rosette-like arrangements of cells are seen. Unable to visualize either budding or asymmetric division of cells by optical microscopy. Though single polar flagellum was observed by electron microscopy (Sly, 1985), neither active motility in wet mount preparation nor diffuse spreading growth on semisolid motility agar plate was demonstrated. Colonies are orange pigmented. Aerobic and facultative photoorganotrophs, possessing bacteriochlorophyll *a*. Acid produced oxidatively from fructose and maltose but not from glucose. Isolated from a fresh water swimming pool. Major polyamine is spermidine (Busse et al., 1999; Takeuchi et al., 2001).

The mol% G + C of the DNA is: 64.5 (HPLC).

Type strain: ACM 2507, ATCC 35951, DSM 3183, EY 4220, IFO15649, JCM 10396, LMG 17322, NCIMB 12085.

GenBank accession number (16S rRNA): Y13774.

18. ***Sphingomonas parapaucimobilis*** Yabuuchi, Yano, Oyaizu,

*Editorial Note: *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997, 568 emend. Hiraishi, Kuraishi and Kawahara 2000a, 1117 is a junior objective synonym of *S. natatoria* (Yabuuchi et al., 2002).

TABLE BXII.α.87. Susceptibilities of type strain of 33 *Sphingomonas* species against 36 antibacterial agents determined by Kirby–Bauer method^a

Antibiotic (mg/disk)	Clinical, hospital, laboratory							PP		Plant				Soil and sediments			
	<i>S. paucimobilis</i> EY 2395 ^T	<i>S. adhaesiva</i> EY 4215 ^T	<i>S. capsulata</i> EY 4216 ^T	<i>S. echinoides</i> EY4340 ^T	<i>S. parapaucimobilis</i> EY 4213 ^T	<i>S. sanguinis</i> EY 2397 ^T	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. melonis</i> EY 4350 ^T	<i>S. suberifaciens</i> EY 4204 ^T	<i>S. asaccharolytica</i> EY 4229 ^T	<i>S. mali</i> EY 4341 ^T	<i>S. pruni</i> EY 4228 ^T	<i>S. rosea</i> EY 4227 ^T	<i>S. roseiflava</i> EY4345 ^T	<i>S. macroglabridus</i> EY4304 ^T	<i>S. truperi</i> EY 4218 ^T	<i>S. herbicidovorans</i> EY4344 ^T
Doxycycline (10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline (30)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Minocycline (30)	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Amikacin (30)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AMPC/CVA (20/10)	S	S	S	S	S	S	S	S	S	S	S	R	S	S	IM	S	S
Gentamicin (10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Panipenem (10)	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	IM	S
Imipenem (10)	S	S	S	S	S	IM	S	S	S	S	S	S	S	S	S	S	S
Dibekacin (30)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sparfloxacin (5)	S	S	IM	R	S	S	S	S	S	IM	S	S	R	S	S	S	S
Clarithromycin (15)	S	S	S	S	S	S	S	S	S	IM	S	R	S	S	S	S	S
Levofloxacin (5)	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S
Cefotaxime (30)	S	S	S	S	R	R	S	R	S	S	S	IM	S	S	R	IM	S
Meropenem (10)	S	S	S	S	S	S	S	S	S	IM	S	S	R	S	R	S	S
Erythromycin (15)	S	R	IM	R	S	S	IM	IM	S	S	R	R	IM	S	S	IM	S
Cefaclor (30)	S	S	S	S	R	R	S	R	S	S	S	R	S	S	R	S	R
Ofloxacin (5)	S	IM	R	S	S	S	S	S	S	R	S	R	R	S	S	IM	S
Tosufloxacin (5)	S	IM	R	R	S	S	IM	S	S	IM	S	R	S	S	S	S	S
Ceftazidime (30)	S	S	S	S	R	R	S	R	S	S	R	S	R	S	R	IM	S
Amoxicillin (25)	S	S	S	S	R	R	S	R	S	S	S	R	R	S	R	S	R
Ciprofloxacin (5)	S	IM	R	S	S	S	S	S	S	R	R	IM	R	S	R	IM	S
Ampicillin (10)	S	S	S	S	R	R	S	R	S	S	S	R	R	S	R	S	R
Polymyxin B (300)	IM	R	IM	R	IM	R	S	R	S	R	R	R	R	S	S	R	S
Sulfamethoxazole-trimethoprim (23.75/1.25)	S	R	S	IM	S	S	S	S	S	S	S	R	R	S	S	R	R
Flomoxef (30)	S	R	S	S	R	R	R	R	S	R	R	IM	R	S	R	R	S
Roxithromycin (15)	S	R	R	R	S	S	R	IM	IM	IM	R	R	IM	S	IM	R	S
Cefmetazole (30)	R	IM	S	S	R	R	R	R	S	S	R	R	R	S	R	R	S
Cefoperazone (75)	S	S	IM	S	R	R	S	R	S	R	R	R	R	R	R	R	R
Penicillin (10)	S	R	S	S	R	R	S	R	S	S	R	R	R	R	R	R	R
Carumonam (30)	S	R	R	R	R	R	R	R	S	S	R	S	S	IM	R	R	R
Piperacillin (100)	R	IM	IM	S	R	R	R	R	S	S	R	R	R	R	R	R	R
Norfloxacin (10)	IM	R	R	R	R	S	IM	IM	R	R	R	R	R	S	S	R	S
Aztreonam (30)	IM	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	IM
Moxalactam (30)	R	R	IM	S	R	R	R	R	S	IM	R	IM	R	R	R	R	R
Cefazolin (30)	R	R	S	S	R	R	R	R	S	R	R	R	R	S	R	R	R
Trimethoprim (5)	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R
Summary:																	
R	5	12	8	9	16	16	9	17	3	9	15	19	21	5	16	14	10
%R	14	33	22	25	44	44	25	47	8	25	42	53	58	14	44	39	28
S + IM	31	24	28	27	20	20	27	19	33	27	21	17	15	31	20	22	26

^aAbbreviations: PP, plant pathogenic; AMPC, amoxicillin; CVA, clavulanic acid; S, susceptible; IM, intermediate; R, resistant.

Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116.)

pa.ra.pau.ci.mo.bi'lis. Gr. prep. *para* alongside of, resembling; M.L. *paucimobilis* specific epithet of *Sphingomonas paucimobilis*; M.L. gen. n. *parapaucimobilis* intended to mean like the species of *S. paucimobilis*.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Cellular and colonial characteristics are similar to those of *S. paucimobilis*. Glucuronosyl ceramide (SGL-1)

is present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major component of polyamine is homospermidine (Hamana and Matsuzuki, 1993; Takeuchi et al., 1995; Takeuchi et al., 2001); Segers et al. (1994) reported production of spermidine. Isolated from human urine.

The mol% G + C of the DNA is: 64–65 (HPLC).

Type strain: ATCC 51231, CCUG 27291, DSM 7463, EY 4213, GIFU 11387, IFO 15100, JCM 7510, LMG 10923.

GenBank accession number (16S rRNA): X72721.

19. ***Sphingomonas pituitosa*** Denner, Paukner, Kämpfer, Moore, Abraham, Busse, Wanner and Lubitz 2001, 837^{VP}
pi.tu.i.to'sa. L. fem. adj. *pituitosa* slimy.

TABLE BXII.α.87. (cont.)

Antibiotic (mg/disk)	Soil and sediments				Sludge			Fresh water						Sea or mineral water			No. of resistant strains
	<i>S. terrae</i> EY 4207 ^T	<i>S. aromaticivorans</i> EY4296 ^T	<i>S. sfygia</i> EY4297 ^T	<i>S. subterranea</i> EY4298 ^T	<i>S. chungbukensis</i> EY 4375 ^T	<i>S. chlorophenolica</i> EY 4219 ^T	<i>S. cloacae</i> EY 4361 ^T	<i>S. subarctica</i> EY4251 ^T	<i>S. witlichii</i> EY 4224 ^T	<i>S. pituitosa</i> EY 4370 ^T	<i>S. xenophaga</i> EY4343 ^T	<i>S. ursincola</i> EY4250 ^T	<i>S. natalonia</i> EY 4220 ^T	<i>S. alaskensis</i> EY 4374 ^T	<i>S. koreensis</i> EY 4376 ^T	<i>S. taenionensis</i> EY 4377 ^T	
Doxycycline (10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0
Tetracycline (30)	S	S	S	S	S	S	S	S	IM	S	IM	S	S	S	S	S	0
Minocycline (30)	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	2
Amikacin (30)	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	IM	2
AMPC/CVA (20/10)	S	S	S	S	S	S	IM	S	S	S	S	S	S	IM	R	R	3
Gentamicin (10)	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	S	3
Panipenem (10)	S	S	S	S	S	S	S	IM	S	S	S	S	S	S	R	IM	3
Imipenem (10)	S	S	S	S	S	S	R	S	S	R	S	S	S	S	R	S	3
Dibekacin (30)	S	S	S	S	S	S	R	S	S	S	S	R	S	R	R	IM	4
Sparfloxacin (5)	S	R	S	IM	S	S	S	R	R	S	S	IM	S	S	IM	S	5
Clarithromycin (15)	R	S	S	S	S	R	R	S	R	IM	S	S	S	S	R	R	6
Levofloxacin (5)	S	IM	R	R	S	S	S	R	R	S	S	IM	S	S	S	S	6
Cefotaxime (30)	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	7
Meropenem (10)	IM	S	S	S	S	S	R	IM	S	R	S	S	S	R	R	R	7
Erythromycin (15)	IM	IM	S	IM	S	R	S	IM	R	S	IM	IM	S	IM	R	R	8
Cefaclor (30)	IM	M	S	IM	S	S	S	R	S	IM	S	S	S	R	R	R	10
Ofloxacin (5)	S	R	R	R	S	S	S	R	R	S	S	IM	S	S	R	IM	10
Tosufloxacin (5)	IM	R	R	R	S	S	S	R	R	S	IM	IM	R	S	R	IM	10
Ceftazidime (30)	S	S	S	S	S	S	R	IM	S	R	S	S	S	R	R	R	11
Amoxicillin (25)	S	S	S	S	S	S	R	S	S	R	R	S	S	IM	R	IM	11
Ciprofloxacin (5)	S	R	R	R	S	S	S	R	R	IM	S	S	S	S	R	IM	11
Ampicillin (10)	S	S	S	S	R	S	R	S	S	R	R	S	S	R	R	R	14
Polymyxin B (300)	R	S	R	R	S	S	S	S	IM	R	S	IM	IM	S	R	S	14
Sulfamethoxazole-trimethoprim (23.75/1.25)	R	IM	R	R	R	S	S	R	R	IM	R	R	S	R	R	R	A6
Flomoxef (30)	R	S	S	S	S	S	R	IM	S	R	S	IM	IM	R	R	R	16
Roxithromycin (15)	R	IM	S	S	S	R	R	R	R	IM	IM	R	S	R	R	R	16
Cefmetazole (30)	R	R	S	R	S	S	S	R	S	S	S	IM	IM	R	R	R	17
Cefoperazone (75)	S	S	S	S	R	S	R	S	IM	R	R	S	S	IM	R	R	17
Penicillin (10)	S	S	S	R	R	S	R	S	S	S	R	S	S	R	R	R	18
Carumonam (30)	R	S	S	S	R	R	S	IM	S	S	IM	R	R	S	R	R	18
Piperacillin (100)	S	S	S	S	R	S	R	S	S	R	R	S	S	R	R	R	19
Norfloxacin (10)	IM	R	R	R	S	S	R	R	R	R	S	IM	S	S	R	R	19
Aztreonam (30)	R	IM	S	S	R	R	S	S	R	S	IM	R	IM	R	R	R	21
Moxalactam (30)	R	R	S	R	R	R	S	R	R	S	R	R	R	R	R	R	25
Cefazolin (30)	S	R	S	R	R	R	R	R	S	R	R	R	S	R	R	R	25
Trimethoprim (5)	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	29
Summary:																	
R	10	9	8	12	10	8	14	13	13	12	9	9	4	18	31	21	
%R	28	25	22	33	28	22	39	36	36	33	25	25	11	50	86	58	
S + IM	26	27	28	24	26	28	22	23	23	24	16	27	32	18	5	15	

^aAbbreviations: PP, plant pathogenic; AMPC, amoxicillin; CVA, clavulanic acid; S, susceptible; IM, intermediate; R, resistant.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Cells are 0.4–0.75 × 1.0–3.0 μm. Motile by a single polar flagellum. Strictly aerobic. Colonies are deep yellow. Poly-β-hydroxybutyrate is accumulated (Denner et al., 2001). Glucuronosyl ceramide (SGL-1) is present. Major polyamine is homospermidine (Denner et al., 2001). Isolated from the water of a eutrophic fountain in Vienna, Austria, in which an algal bloom was observed.

The mol% G + C of the DNA is: 64.5 (HPLC).

Type strain: EDIV, CIP 106154, DSM 13101, EY 4370.

GenBank accession number (16S rRNA): AJ243751.

20. **Sphingomonas pruni** Takeuchi, Sakane, Yanagi, Yamasato, Hamana and Yokota 1995, 340^{VP}
pru'ni. M.L. gen. n. *Prunus* genus of peach; L, gen. n. *pruni* of peach (*Prunus persica*), the source of the organism.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Neither active motility in wet mount preparation nor diffuse spreading growth on semisolid motility agar plate was observed. Colonies are grayish white. Glucuronosyl ceramide (SGL-1) is present, but not SGL-1' (Fig. BXII.α.85). Major polyamine is homospermidine (Takeuchi

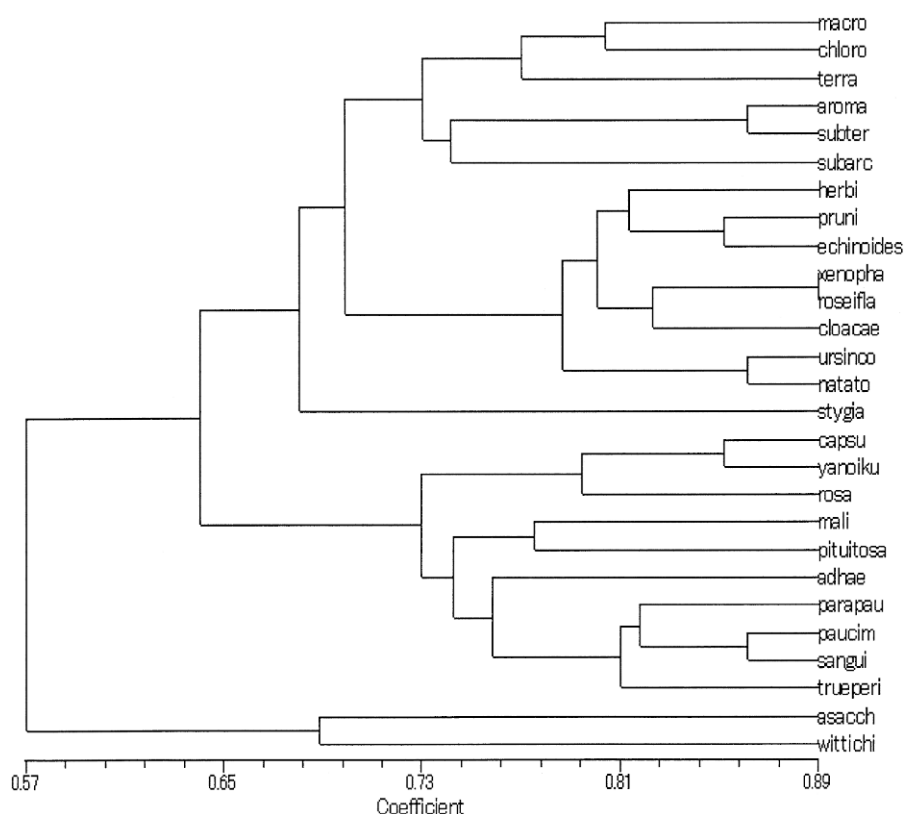


FIGURE BXII.α.95. Numerical analysis of 106 features of type strains of 27 *Sphingomonas* species. Numerical analysis was done by using NTSYS-pc Version 2 program package (Applied Biostatistics Inc. Setauket, NY). Coefficient was calculated by Simple Matching, and the dendrogram was drawn by UPGMA (Sneath and Sokal, 1973). Name of *Sphingomonas* species (new genus by Takeuchi et al., 2001): macro = *S. macrogoltabidus* (*Sphingopyxis*); chloro = "*S. chlororaphis*" (*Sphingobium*); terra = *S. terrae* (*Sphingopyxis*); aroma = *S. aromaticivorans* (*Sphingomonas*); subter = *S. subterranea* (*Sphingomonas*); subarc = *S. subarctica* (*Sphingomonas*); herbi = *S. herbicidovorans* (*Sphingobium*); pruni = *S. pruni* (*Sphingomonas*); echinoides = *S. echinoides* (*Sphingomonas*); xenopha = *S. xenophaga*; roseifla = *S. roseiflava* (*Sphingomonas*); cloacae = *S. cloacae*; ursincola = *S. ursincola* (*Blastomonas*); natato = *S. natatoria* (*Blastomonas*); stygia = *Sphingomonas stygia* (*Novosphingobium*); capsu = *S. capsulata* (*Novosphingobium*); yanoiku = *S. yanoikuyae* (*Sphingobium*); rosa = *S. rosa* (*Novosphingobium*); mali = *S. mali* (*Sphingomonas*); pituitosa = *S. pituitosa*; adhae = *S. adhaesiva* (*Sphingomonas*); parapau = *S. parapaucimobilis* (*Sphingomonas*); sangui = *S. sanguinis* (*Sphingomonas*); trueperi = *S. trueperi*; asacch = *S. asaccharolytica* (*Sphingomonas*); wittichi = *S. wittichii* (*Sphingomonas*).

et al., 1995; Takeuchi et al., 2001). Isolated from the roots of *Prunus persica* (peach) in Tsukuba City, Japan.

The mol% G + C of the DNA is: 65.4 (HPLC).

Type strain: Y-250, DSM 10566, EY 4228, IFO 15498, JCM 10277, LMG 18380.

GenBank accession number (16S rRNA): D28568, Y09637.

21. ***Sphingomonas rosa*** Takeuchi, Sakane, Yanagi, Yamasato, Hamana and Yokota 1995, 340^{VP*}
ro'sa. M.L. n. *rosa* rose, the source of the organism.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Neither active motility in wet mount preparation nor diffuse spreading growth on semisolid motility agar plate was observed. Colonies are light yellow. Glucuronosyl ceramide (SGL-1) is present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major polyamine is sper-

midine (Takeuchi et al., 1995; Takeuchi et al., 2001). Isolated from the hairy roots of *Rosa* spp. (rose).

The mol% G + C of the DNA is: 64.7–65.0 (HPLC).

Type strain: ATCC 51837, DSM 7285, EY 4227, IFO 15208, JCM 10276, LMG 17328, NCPPB 2661.

GenBank accession number (16S rRNA): D13945.

22. ***Sphingomonas roseiflava*** Yun, Shin, Hwang, Kuraishi, Sugiyama and Kawahara 2000b, 1415^{VP} (Effective publication: Yun, Shin, Hwang, Kuraishi, Sugiyama and Kawahara 2000a, 17) (*Sphingomonas roseoflava* (sic) Yun, Shin, Hwang, Kuraishi, Sugiyama and Kawahara 2000b, 1415.)
ro.se.i.flu'va. L. adj. *roseus* rose colored; L. adj. *flavus* yellow; N.L. fem. adj. *roseiflava* rose-yellow.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Nonmotile rod. Colonies are orange. Glucuronosyl ceramide (SGL-1) is present but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Two types of sphingoglycolipid are present: one contains glucuronic acid, and the other contains glucuronic acid, glucosamine, and hex-

*Editorial Note: *Novosphingobium rosa* (Takeuchi et al. 1995) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. rosa* (Yabuuchi et al., 2002).

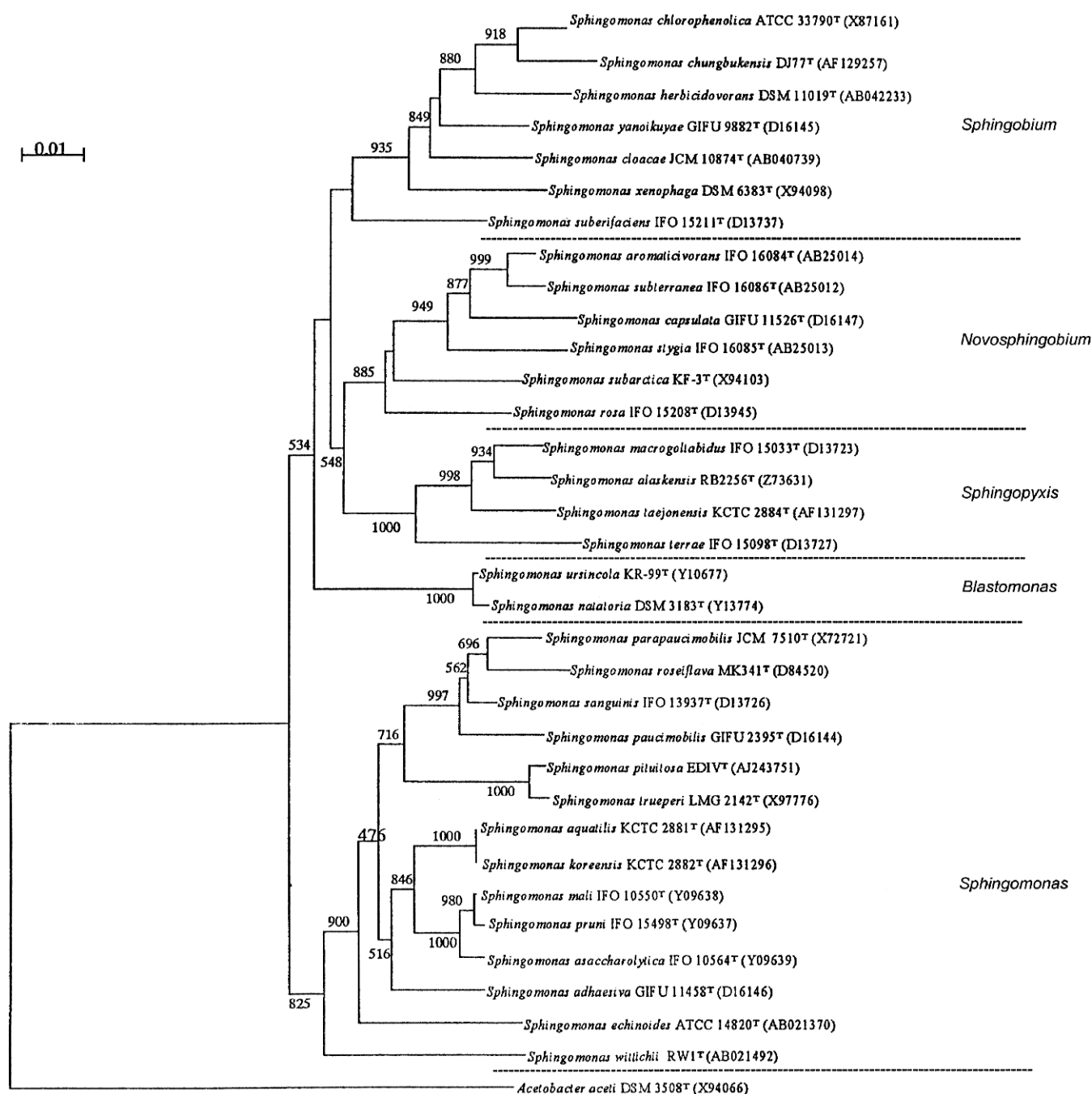


FIGURE BXII.α.96. Dendrogram based on the results of phylogenetic analysis of 16S rDNA sequence of type strains of 33 *Sphingomonas* species. Nucleotide substitution rates (K_{nuc}) (Kimura, 1980) were determined by using the CLUSTAL W program (Thompson et al., 1994). Neighbor-joining method (Saitou and Nei, 1987) was used to reconstruct a phylogenetic tree from the distance matrices by the NJPLOT written by Manolo Gouy (Laboratoire de Biometrie, Univ. Lyon, Villeurbanne, France). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. To evaluate the topology of the phylogenetic tree, a bootstrap analysis was performed with 1000 bootstrapped trials. Bootstrap values are indicated at the branching points. Clusters containing *Sphingomonas* species assigned by Takeuchi et al., 2001 to the genera *Sphingobium*, *Sphingopyxis*, and *Novosphingobium* as well as the closely related *Blastomonas* species are indicated on the right.

oses (Yun et al., 2000a). Frequently isolated from the ear of the plant *Setaria viridis*.

The mol% G + C of the DNA is: 66–68 (HPLC).

Type strain: MK3412, EY 4345, IAM 14823.

GenBank accession number (16S rRNA): D84520.

23. *Sphingomonas sanguinis* Takeuchi, Kawai, Shimada and

Yokota 1993b, 864^{VP} (Effective publication: Takeuchi, Kawai, Shimada and Yokota 1993a, 237) (*Sphingomonas sanguinis* (sic) Takeuchi, Kawai, Shimada, and Yokota 1993b, 864.) *san'gui.nis*. L. gen. n. *sanguinis* of the blood.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87,

TABLE BXII.α.88. Oxidative acid production of the type strains of 38 species of *Sphingomonadaceae* in Bacto-Oxidation–Fermentation (OF), Cystine–tryptone agar, or Marine OF media^{a,b}

Carbohydrates	<i>Sphingomonas paucimobilitis</i> EY 2595 ^T (a) ^{c,d}	<i>Sphingomonas alaskensis</i> EY 4374 ^T (b)	<i>Sphingomonas adhaesiva</i> EY 4215 ^T (a)	<i>Sphingomonas aquatilis</i> KCTC 2881 ^T (a) ^e	<i>Sphingomonas aromaticivorans</i> EY 4296 ^T (b)	<i>Sphingomonas asaccharolytica</i> EY 4229 ^T (a)	<i>Sphingomonas capsulata</i> EY 4216 ^T (b)	<i>Sphingomonas chlorophenolica</i> EY 4219 ^T (a)	<i>Sphingomonas chungbukensis</i> EY 4375 ^T (a)	<i>Sphingomonas cloacae</i> EY 4361 ^T (a)	<i>Sphingomonas echinoides</i> EY 4340 ^T (a)	<i>Sphingomonas herbicidovorans</i> EY 4344 ^T (b)	<i>Sphingomonas korensis</i> EY 4376 ^T (a)	<i>Sphingomonas macroglabridus</i> EY 4304 ^T (b)	<i>Sphingomonas mali</i> EY 4341 ^T (a)	<i>Sphingomonas natatoria</i> EY 4220 ^T (b)	<i>Sphingomonas parapaucimobilitis</i> EY 4213 ^T (a)	<i>Sphingomonas pituitosa</i> EY 4370 ^T (a)	<i>Sphingomonas pruni</i> EY 4228 ^T (a)
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L-Arabinose	+	–	+	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–
Cellobiose	–	–	–	–	+	+	–	+	–	–	–	+	+	–	–	–	+	+	+
Dulcitol	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	+	–	–	–
Ethanol (3%)	+	–	+	–	–	NG	+	–	–	–	–	–	NG	–	+	–	+	–	–
Fructose	+	–	+	–	+	+	+	–	–	–	+	–	+	–	+	+	+	–	+
Galactose	+	–	+	–	–	–	+	–	+	–	–	+	–	+	+	–	+	+	–
Glucose	–	–	+	–	+	+	+	+	–	–	+	+	+	–	+	–	+	+	+
Glycerol	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Inulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lactose	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	+	+	+
Maltose	–	–	–	–	+	+	+	+	+	+	+	–	–	–	+	–	+	+	+
Mannitol	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Mannose	+	–	+	–	+	+	+	+	–	–	+	–	–	–	+	–	+	+	+
Melezitose	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	+	–	–
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–
Raffinose	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	+	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–
D-Ribose	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	–	+	+	–	+	–	+	+	–	–	+	–	–	+	–	+	+	+	–
Trehalose	–	+	+	–	–	–	–	–	–	–	+	–	+	+	+	–	–	–	–
Xylose	+	–	+	–	–	–	+	–	–	–	+	+	+	–	+	–	–	–	–
Total No. of carbohydrates oxidized	6	2	12	–	10	8	12	14	3	1	10	5	6	7	12	3	14	9	7

^a *Sphingomonas suberifaciens* EY 4202^T was omitted because of no growth on any of three media.^b Symbols: +, positive reaction within 4 d; –, negative reaction for 4 weeks; NG, no growth.^c EY, Eiko Yabuuchi.^d (a) Bacto-Oxidation–Fermentation (OF) basal medium; (b) Bacto-Cystine–tryptone agar (CTA) medium; (c) Bacto-Marine Oxidation–Fermentation (MOF) medium.^e Data from Lee et al. (2001).

BXII.α.88, and BXII.α.89, with the following additional information. Rods. Motile by a single polar flagellum. Colonies are deep yellow. A thin-layer chromatogram of the alkali-stable lipids is shown in Fig. BXII.α.85. Major component of polyamine is homospermidine (Hamana and Matsuzaki, 1993; Segers et al., 1994; Takeuchi et al., 1995; Takeuchi et al., 2001); Segers et al. (1994) reported trace amounts of spermidine. PEG 4000 and PEG 6000 are not assimilated (Takeuchi et al., 1993a). Isolated from blood.

The mol% G + C of the DNA is: 61.8 (HPLC).

Type strain: ATCC 51382, CDC B4562, CIP 104197, DSM 13885, EY 2397, GIFU 2397, IFO 13937, JCM 7514, LMG 17325, NCTC 11032.

GenBank accession number (16S rRNA): D13726.

24. *Sphingomonas stygia* Balkwill, Drake, Reeves, Fredreckson,

White, Ringelberg, Chandler, Romine, Kennedy and Spadoni 1997, 199^{VP*}

stygi.a. L. masc. n. *Styx* underworld river in classical Greek mythology; L. fem. adj. *stygia* pertaining to the underworld, subterranean.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Nonmotile rods. Colonies are light yellow. Glucuronosyl ceramide (SGL-1) is present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major component of polyamine is spermidine (Busse et al., 1999; Takeuchi et

*Editorial Note: *Novosphingobium stygium* (Balkwill et al. 1997) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. stygia* (Yabuuchi et al., 2002).

TABLE BXII.α.88. (cont.)

Carbohydrates	<i>Sphingomonas rosa</i> EY 4227 ^T (a)	<i>Sphingomonas roseiflava</i> EY 4345 ^T (b)	<i>Sphingomonas sanguinis</i> EY 2397 ^T (a)	<i>Sphingomonas stygia</i> EY 4297 ^T (a)	<i>Sphingomonas subarctica</i> EY 4251 ^T (a)	<i>Sphingomonas subterranea</i> EY 4298 ^T (b)	<i>Sphingomonas taenionensis</i> EY 4377 ^T (a)	<i>Sphingomonas terrae</i> EY 4207 ^T (b)	<i>Sphingomonas trueperi</i> EY 4218 ^T (a)	<i>Sphingomonas ursincola</i> EY 4250 ^T (a)	<i>Sphingomonas wittichii</i> EY 4224 ^T (a)	<i>Sphingomonas xenophaga</i> EY 4343 ^T (b)	<i>Sphingomonas yanoikuyae</i> EY 4208 ^T	<i>Sphingomonas melonis</i> EY 4350 ^T (a)	<i>Erythrobacter longus</i> EY 4203 ^T	<i>Erythrobacter litoralis</i> EY 4222 ^T	<i>Erythromicrobium ramosum</i> EY 4223 ^T	<i>Porphyrobacter neustonensis</i> EY 4349 ^T (b)	<i>Porphyrobacter tepidarius</i> EY 4231 ^T (a)
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–
L-Arabinose	+	–	–	–	+	+	–	–	+	–	–	+	+	+	–	–	–	–	–
Cellobiose	+	+	–	+	–	–	–	+	–	+	–	+	–	+	+	–	+	–	+
Dulcitol	+	–	–	–	+	–	–	+	–	+	–	+	–	–	–	–	–	–	+
Ethanol (3%)	NG	–	+	NG	–	NG	NG	–	–	–	–	–	–	–	–	+	–	–	–
Fructose	+	+	–	–	+	+	–	–	–	–	+	–	–	+	–	–	–	+	+
Galactose	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	+
Glucose	+	+	+	–	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–
Glycerol	–	–	+	–	–	–	–	–	–	–	+	–	–	+	–	–	–	+	+
Inositol	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Inulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Lactose	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Maltose	+	+	–	+	+	–	+	+	+	+	+	–	–	+	+	–	–	+	+
Mannitol	–	–	–	+	+	–	–	+	–	+	+	–	–	–	–	–	–	–	+
Mannose	+	+	+	–	+	–	+	+	+	+	–	–	–	+	–	–	–	+	+
Melezitose	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Melibiose	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Raffinose	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Rhamnose	+	–	–	–	+	–	–	+	–	–	+	–	–	+	–	–	–	–	+
D-Ribose	–	–	–	+	+	–	–	+	–	+	+	–	–	–	–	–	–	+	+
Salicin	+	–	–	–	–	–	–	+	–	–	+	+	–	–	+	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+
Sucrose	+	+	–	+	+	–	–	–	+	+	–	–	–	+	+	–	–	+	–
Trehalose	+	+	–	–	+	–	–	+	+	–	–	–	+	+	+	–	–	–	–
Xylose	+	+	+	–	+	+	–	–	+	–	–	–	+	+	+	–	–	–	–
Total No. of carbohydrates oxidized	15	11	8	8	15	4	4	9	11	7	11	4	3	12	7	4	1	7	11

^a *Sphingomonas suberifaciens* EY 4202^T was omitted because of no growth on any of three media.

^b Symbols: +, positive reaction within 4 d; –, negative reaction for 4 weeks; NG, no growth.

^c EY, Eiko Yabuuchi.

^d (a) Bacto-Oxidation–Fermentation (OF) basal medium; (b) Bacto-Cystine–tryptone agar (CTA) medium; (c) Bacto-Marine Oxidation–Fermentation (MOF) medium.

^e Data from Lee et al. (2001).

al., 2001). Isolated from saturated Atlantic coastal plain terrestrial subsurface sediments.

The mol% G + C of the DNA is: 65.4 (HPLC).

Type strain: B0712, ATCC 700280, DSM 12425, EY 4297, JCM 16085, SMCC B0712.

GenBank accession number (16S rRNA): U20775.

25. ***Sphingomonas subarctica*** Nohynek, Nurmiäho-Lassila, Suhonen, Busse, Mohammadi, Hantula, Rainey and Salkinoja-Salonen 1996a, 1053^{VP*}

sub.arctic M.L. adj. *subarcticus* below the arctic, because the organism was isolated from a subarctic area, Finland.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional in-

formation. Though an electron micrograph of a cell with four flagella arranged peritrichously appeared (Nohynek et al., 1996a), neither active motility in wet mount preparation nor diffuse spreading growth on semisolid motility agar plate were observed. Colonies are light yellow. Glucuronosyl ceramide (SGL-1) is present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Strain KF1 degrades 2,4,6-tri- and 2,3,4,6-tetrachlorophenols but not pentachlorophenol. Major polyamine is spermidine (Nohynek et al., 1996a; Takeuchi et al., 2001). Isolated from the biofilm of an activated sludge reactor.

The mol% G + C of the DNA is: 65–67 (*T_m*).

Type strain: KF1, DSM 10700, EY 4251, HAMBI 2110, JCM 10398.

GenBank accession number (16S rRNA): X94102.

26. ***Sphingomonas suberifaciens*** (van Bruggen, Jochimsen and Brown 1990) Yabuuchi, Kosako, Naka, Suzuki and Yano

*Editorial Note: *Novosphingobium subarcticum* (Balkwill et al. 1997) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. subarctica* (Yabuuchi et al., 2002).

TABLE BXII.α.89. Results of API 20NE of type strains of 34 *Sphingomonas* species^a

Substrate or test ^b	<i>S. paucimobilis</i> EY 2895 ^T	<i>S. adhaesiva</i> EY 4215 ^T	<i>S. alaskensis</i> EY 4374 ^T	<i>S. aquatilis</i> KCTC 2881 ^{Tc}	<i>S. aromaticivorans</i> EY 4296 ^T	<i>S. asaccharolytica</i> EY 4229 ^T	<i>S. capsulata</i> EY 4216 ^T	<i>S. chlorophenolica</i> EY 4219 ^T	<i>S. chungbukensis</i> EY 4375 ^T	<i>S. cloacae</i> EY 4361 ^T	<i>S. echinoides</i> EY 4340 ^T	<i>S. herbicidovorans</i> EY 4344 ^T	<i>S. koreensis</i> EY 4376 ^T	<i>S. macrogoltabidus</i> EY 4304 ^T	<i>S. mali</i> EY 4341 ^T	<i>S. melonis</i> EY 4150 ^T	<i>S. natatoria</i> EY 4220 ^T
<i>p</i> -Nitro-β-D-galactopyranoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Assimilation of:																	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potassium gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>n</i> -Caprate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adipate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DL-Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylacetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

^aFor symbols see standard definitions.^bAll 26 strains positive for hydrolysis of esculin and Zinc dust test in negative NO₂ test. All 26 strains negative for NO₂ from NO₃, indole from tryptophan, acid from glucose, arginine dihydrolase activity, hydrolysis of urea and gelatin.^cData from Lee et al. (2001).^dPositive reaction after more than 5 d.

1999b, 935^{VP} (Effective publication: Yabuuchi, Kosako, Naka, Suzuki and Yano 1999a, 347) (*Rhizomonas suberifaciens* van Bruggen, Jochimsen and Brown 1990, 186.)* *su.be.ri.faci.ens*. L. gen. n. *suberus* of cork, corky; L. part. adj. *faciens* making, producing; M.L. part. adj. *suberifaciens* corky making.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.87, and BXII.α.89, with the following additional information. Cells of the type strain are thin and short straight rods. Actively motile in wet mount preparation. Spreading growth occurs on semi-solid (0.3%) agar medium. Colonies on agar plate of ATCC medium 1700 (*Rhizobium* medium) after 24 h at 26°C are colorless and translucent. Unable to determine acid production from carbohydrates, because neither OF nor CTA medium supports the growth of the organism. Glucuronosyl ceramide (SGL-1) is present but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major polyamine is spermidine (Takeuchi et al., 1995; Takeuchi et al., 2001). Isolated from the corky roots of lettuce (*Lactuca sativa*).

The mol% G + C of the DNA is: 59.0 (HPLC).

Type strain: Ca1, ATCC 49355, DSM 7465, EY 4204, IFO 15211, JCM 8521, NCPPB 3629.

GenBank accession number (16S rRNA): D13737.

27. ***Sphingomonas subterranea*** Balkwill, Drake, Reeves, Fredrickson, White, Ringelberg, Chandler, Romine, Kennedy and Spadoni 1997, 199^{VP**}

sub.ter.ra'ne.a. L. fem., adj. *subterranea* underground, subterranean.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Colonies are deep yellow. Glucuronosyl ceramide (SGL-1) is present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major component of polyamine is spermidine (Takeuchi et al., 2001). Isolated from saturated Atlantic coastal plain terrestrial subsurface sediments.

The mol% G + C of the DNA is: 60.0 (HPLC).

Type strain: B0478, ATCC 700279, DSM 12447, EY 4298, JCM 16086, SMCC B0478.

GenBank accession number (16S rRNA): U20773.

28. ***Sphingomonas taejonensis*** Lee, Kook Shin, Yoon, Takeuchi, Pyun and Park 2001, 1497^{VP}
tae.jon.en'sis. N.L. fem. adj. *taejonensis* of Taejon, Korea, the geographical origin of the species.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Motile by a single polar flagellum. Colonies are pale yellow. Sphingolipid is present, though no details have been reported. Isolated from natural mineral water from Taejon City, Korea.

The mol% G + C of the DNA is: 63 (HPLC).

Type strain: JSS54, EY 4377, JCM 11457, KCTC 2884, KCM 41068.

GenBank accession number (16S rRNA): AF131297.

*Editorial Note: The generic name *Rhizomonas* Orla-Jensen 1909 has been placed on the List of Rejected Generic Names, Opinion 14 of the Judicial Commission, Lapage et al. (1992).

**Editorial Note: *Novosphingobium subterraneum* (Balkwill et al. 1997) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. subterranea* (Yabuuchi et al., 2002).

TABLE BXII.α.89. (cont.)

Substrate or test ^b	<i>S. parapaucimobilis</i> EY 4213 ^T	<i>S. pituitosa</i> EY 4370 ^T	<i>S. pruni</i> EY 4228 ^T	<i>S. rosa</i> EY 4227 ^T	<i>S. roseiflava</i> EY 4345 ^T	<i>S. sanguinis</i> EY 2397 ^T	<i>S. stygia</i> EY 4297 ^T	<i>S. subarctica</i> EY 4251 ^T	<i>S. suberifaciens</i> EY 4204 ^T	<i>S. subterranea</i> EY 4298 ^T	<i>S. taenionensis</i> EY 7377 ^T	<i>S. terrae</i> EY 4377 ^T	<i>S. trueperi</i> EY 4218 ^T	<i>S. ursincola</i> EY 4250 ^T	<i>S. uittichii</i> EY 4224 ^T	<i>S. xenophaga</i> EY 4343 ^T	<i>S. yanoikuyae</i> EY 4208 ^T
<i>p</i> -Nitro-β-D-galactopyranoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Assimilation of:																	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potassium gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
n-Caprinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adipate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DL-Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylacetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

^aFor symbols see standard definitions.^bAll 26 strains positive for hydrolysis of esculin and Zinc dust test in negative NO₂ test. All 26 strains negative for NO₂ from NO₃, indole from tryptophan, acid from glucose, arginine dihydrolase activity, hydrolysis of urea and gelatin.^cData from Lee et al. (2001).^dPositive reaction after more than 5 d.

29. **Sphingomonas terrae** Takeuchi, Kawai, Shimada and Yokota 1993b, 864^{VP} (Effective publication: Takeuchi, Kawai, Shimada and Yokota 1993a, 236.)*
ter'rae. L. n. *terra* earth; M.L. gen. n. *terrae* of the earth.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Rods with round ends. Motile by a single polar flagellum. Colonies are deep yellow. PEG 6000 is assimilated in a mixed culture. Both glucuronosyl ceramide (SGL-1) and galacturonosyl ceramide (SGL-1') are present (Fig. BXII.α.85). Polyamines reported include spermidine alone (Takeuchi et al., 1995; Takeuchi et al., 2001) and homospermidine together with a trace of spermidine (Segers et al., 1994). Isolated from active sludge.

The mol% G + C of the DNA is: 63.0–64.9 (HPLC).

Type strain: E-1-A, ATCC 51381, CIP 104198, DSM 8831, EY 4207; IFO 15098, JCM 10195, LMG 17326.

GenBank accession number (16S rRNA): D13727.

30. **Sphingomonas trueperi** Kämpfer, Denner, Meyer, Moore and Busse 1997, 579^{VP}
true'peri. L. gen. n. *trueperi* of Trüper; named in honor of the German microbiologist Hans G. Trüper in recognition of his numerous contributions to the taxonomy of the *Proteobacteria*.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional in-

formation. Motile with peritrichous flagella. Colonies are light yellow. Able to grow in the presence of the 10% sodium chloride and 0.02% sodium azide (Kämpfer et al., 1997). Reported polyamines include homospermidine together with spermidine (Busse and Auling, 1988) or homospermidine alone (Takeuchi et al., 2001). Glucuronosyl ceramide (SGL-1) present but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Isolated from soil.

The mol% G + C of the DNA is: 65.6 (*T_m*).

Type strain: ATCC 12417, DSM 7225, EY 4218, JCM 10278, LMG 2141, NCIMB 9391.

GenBank accession number (16S rRNA): X97776.

31. **Sphingomonas ursincola** (Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997) Yabuuchi, Kosako Naka, Suzuki and Yano 1999b, 935^{VP} (Effective publication: Yabuuchi, Kosako, Naka, Suzuki and Yano 1999a, 347) (*Erythromonas ursincola* Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177.)*
ur.sin'co.la. M.L. adj. *ursincola* neighbor or compatriot of bears.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Short rod or ovoid cells. Rosettes are not formed. In wet mount preparation, cells seem nonmotile and coccoid. No diffuse spreading growth on semisolid motility agar plate. Unable to visualize either budding or asymmetric division of cells by optical microscopy of Gram-

*Editorial Note: *Sphingopyxis terrae* (Takeuchi et al. 1993a) Takeuchi et al. 2001, 1416 is a junior objective synonym of *S. terrae* (Yabuuchi et al., 2002).

**Editorial Note: *Blastomonas ursincola* (Yurkov et al. 1997) Hiraishi et al. 2000a, 1117 is a junior objective synonym of *S. ursincola* (Yabuuchi et al., 2002).

stained preparations. Aerobic and facultative photoorganotrophic. Colonies are orange brown. Needs 5 d for esculin hydrolysis. Glucuronosyl ceramide (SGL-1) present but not galacturonosyl ceramide (SGL-1'). (Fig. BXII.α.85). Isolated from a freshwater cyanobacterial mat.

The mol% G + C of the DNA is: 64.8 (HPLC).

Type strain: KR-99, DSM 9006, EY 4250, JCM 10397.

GenBank accession number (16S rRNA): Y10677.

32. **Sphingomonas wittichii** Yabuuchi, Yamamoto Terakubo, Okamura, Naka, Fujiwara, Kobayashi, Kosako and Hiraishi 2001, 289^{VP}

wi.tti'chi.i. M.L. gen. n. *wittichii* of Wittich, referring to Rolf Michael Wittich, the German bacteriologist who first isolated this potent metabolizer of dibenzo-*p*-dioxin from the water of the river Elbe and described the metabolism of the compound by this organism.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Rods with rounded ends. Actively motile, with a single polar or subpolar flagellum. Colonies are grayish white and become faintly yellow after incubation for 3 d at 30°C. Both glucuronosyl ceramide (SGL-1) and galacturonosyl ceramide (SGL-1') are present (Fig. BXII.α.85). Polyamines include homospermidine and smaller amounts of spermidine (Busse et al., 1999). Isolated from water of the River Elbe.

The mol% G + C of the DNA is: 67 (T_m).

Type strain: Wittich RW1, DSM 6014, EY 4224, JCM 10273, SMUM 2128.

GenBank accession number (16S rRNA): AB021492.

33. **Sphingomonas xenophaga** Stolz, Schmidt-Maag, Denner, Busse, Egli and Kämpfer 2000, 40^{VP}

xe.no'pha.ga. Gr. adj. *xenos* foreign; Gr. n. *phagos* eater; M.L. fem. adj. *xenophaga* eating foreign (xenobiotic) compounds.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87,

BXII.α.88, and BXII.α.89, with the following additional information. Motile by a polar flagellum. Colonies are deep yellow. Glucuronosyl ceramide (SGL-1) present, but not galacturonosyl ceramide (SGL-1') (Fig. BXII.α.85). Major polyamine is spermidine (Stolz et al., 2000). The type strain was isolated from river water (River Elbe, Germany).

The mol% G + C of the DNA is: 62.1–63.3 (T_m).

Type strain: BN6, DSM 6383, EY 4343.

GenBank accession number (16S rRNA): X94098.

34. **Sphingomonas yanoikuyae** Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990b, 321^{VP} (Effective publication: Yabuuchi, Yano, Oyaizu, Hashimoto, Ezaki and Yamamoto 1990a, 116.)*

ya.no.i.ku'yae. N.L. adj. *yanoikuyae* named in honor of Professor Ikuya Yano, the Japanese bacteriologist who first recognized the second major spot of alkaline-stable glycolipid (SGL-1', now known as galacturonosyl ceramide) on TLC.

The characteristics are as described for the genus and in Tables BXII.α.84, BXII.α.85, BXII.α.86, BXII.α.87, BXII.α.88, and BXII.α.89, with the following additional information. Actively motile with a single polar or subpolar flagellum. Colonies are grayish white, never became definitely yellow after prolonged incubation at room temperature. Both glucuronosyl ceramide (SGL-1) and galacturonosyl ceramide (SGL-1') are present (Fig. BXII.α.85). Major component of polyamine reported as spermidine (Hamana and Matsuzaki, 1993; Segers et al., 1994; Takeuchi et al., 1995; Busse et al., 1999; Takeuchi et al., 2001). Type strain isolated from a hospital specimen (Bruun, 1982); other strains from plant roots.

The mol% G + C of the DNA is: 61.7 (HPLC).

Type strain: ATCC 51230, CCUG 28380, DSM 7462, EY4208, GIFU 9882, IFO 15102, JCM 7371, LMG 11252.

GenBank accession number (16S rRNA): D16145.

*Editorial Note: *Sphingobium yanoikuyae* (Yabuuchi et al. 1990b) Takeuchi et al. 2001, 1415 is a junior objective synonym of *S. yanoikuyae* (Yabuuchi et al., 2002).

Genus II. **Blastomonas** Sly and Cahill 1997, 567^{VP} emend. Hiraishi, Kuraishi and Kawahara 2000a, 1117

LINDSAY I. SLY AND PHILIP HUGENHOLTZ

Blas.to.mo' nas. Gr. n. *blastos* bud shoot; Gr. n. *monas* a unit, monad; M.L. fem. n. *Blastomonas* a budding monad.

Cells are ovoid or rod-shaped and reproduce by budding or asymmetric cell division. They occur singly or in pairs and may form rosette-like aggregates. No stalks and prosthecae are found. Gram negative. Nonsporeforming. Motile by means of polar flagella. Strictly aerobic. Chemoorganotroph and facultative photoorganoheterotroph. No growth occurs under anaerobic conditions in the light. Produces BChl *a*. Colonies and cell suspensions are yellow to orange due to carotenoids. Mesophilic and neutrophilic. Catalase and oxidase positive. Nitrate is not reduced. Acid is not produced in Hugh and Leifson's OF medium. The major whole-cell fatty acid is C_{18:1} ω₉. The major hydroxy-fatty acid is C_{14:0} 2OH. C_{15:0} 2OH and C_{16:0} 2OH are present as minor components. 3-OH fatty acids are absent. Monosaccharide-type glycosphingolipids are present. Ubiquinone-10 is the major respiratory quinone.

Belongs to the *Alphaproteobacteria*. Habitat is fresh water.

The mol% G + C of the DNA is: 65.

Type species: **Blastomonas natatoria** (Sly 1985) Sly and Cahill 1997, 568 emend. Hiraishi, Kuraishi and Kawahara 2000a, 1117 (*Blastobacter natatorius* Sly 1985, 43.)

FURTHER DESCRIPTIVE INFORMATION

The cellular morphology of *Blastomonas* is shown in Fig. BXII.α.97. Some variation in cell shape and arrangement occurs. Cells are usually rod-shaped with straight sides, but may be swollen or wedge-shaped, or have a slightly curved axis (Sly, 1985). In *B. natatoria* each cell has a simple mucilaginous holdfast at its non-reproductive pole, by which it attaches to solid surfaces or other cells to form rosettes (Sly, 1985). Rosette formation in *B. ursincola* has not been reported. Further information on morphological and phenotypic characters may be found in publi-

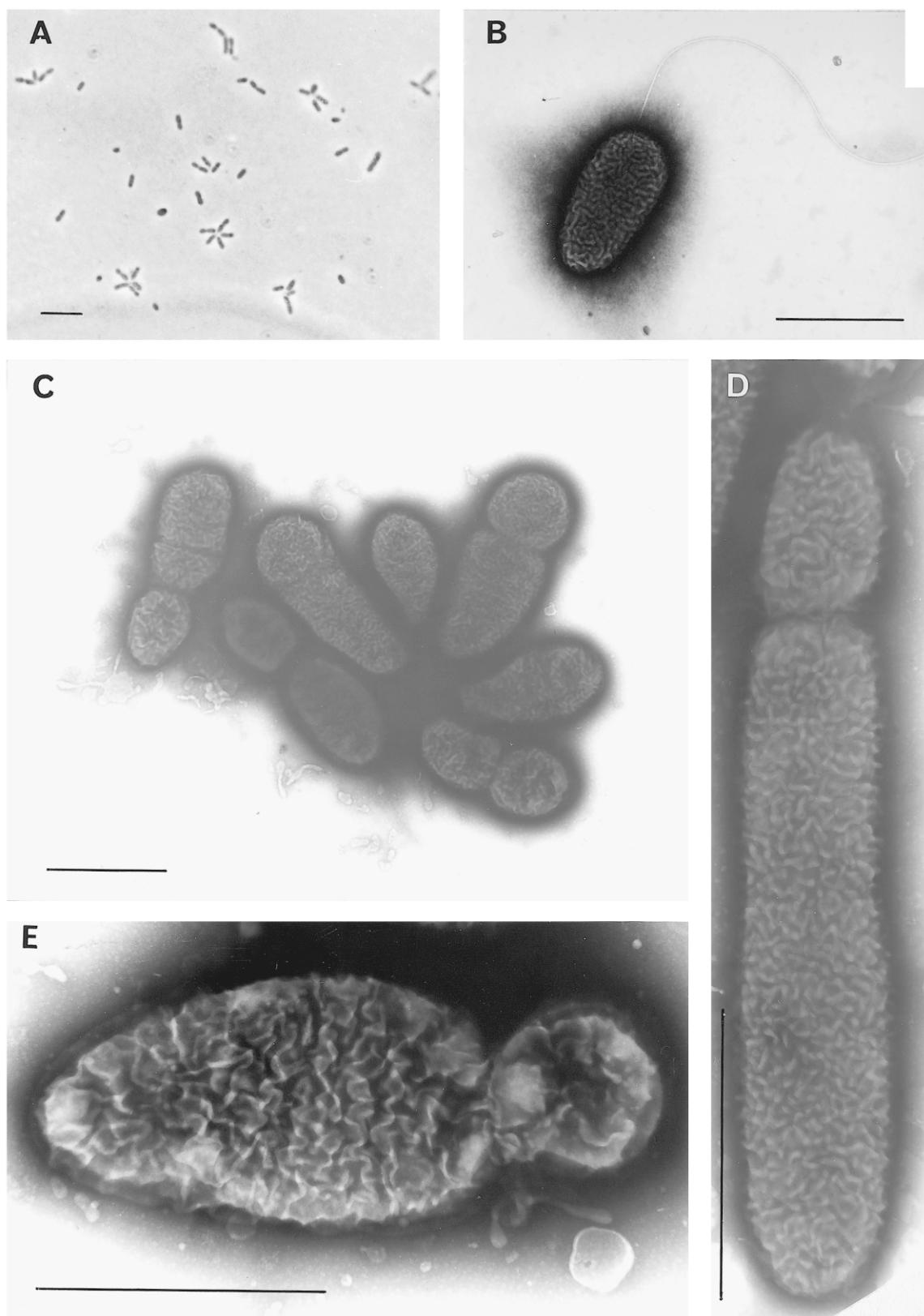


FIGURE BXII.α.97. Morphology of *Blastomonas natatoria* ACM 2057. (A) Photomicrograph showing individual cells and budding cells in rosette formation. Bar = 5 μm . (B) Electron micrograph of a daughter cell (bud) with a single polar flagellum. Bar = 1 μm . (C) Electron micrograph of budding cells in rosette formation. Bar = 1 μm . (D) and (E) Electron micrographs demonstrating different cell morphologies. Bar = 1 μm . (Reprinted with permission from L.I. Sly, *International Journal of Systematic Bacteriology* 35: 40–45, 1985, ©International Union of Microbiological Societies.)

cations by Sly and Hargreaves (1984), Sly (1985), Sly and Cahill (1997), Yurkov et al. (1997), and Hiraishi et al. (2000a). Chemotaxonomic characteristics are described in Sittig and Hirsch (1992), Yurkov et al. (1997), and Hiraishi et al. (2000a).

Considerable research has been undertaken on the photosynthesis characteristics. Bacteriochlorophyll *a* has been detected in the type strains of both *B. natatoria* and *B. ursincola*, but the latter strain produces the photosynthetic pigment in higher amounts (Hiraishi et al., 2000a). *B. ursincola* was shown to have bacteriochlorophyll *a* incorporated into the photochemically active photosynthetic reaction center and core light harvesting (LH1) complexes (Yurkov et al., 1997, 1998a, b). The *puf* genes coding for proteins of the L and M subunits of the photosynthetic reaction center complex and of the LH1 complex have been detected in both species (Hiraishi et al., 2000a). *B. natatoria* and *B. ursincola* show 4–5% difference in their *pufL* and *pufM* gene sequences (Hiraishi et al., 2000a).

B. natatoria and *B. ursincola* contain carotenoid pigments in addition to the bacteriochlorophyll *a* pigment. Carotenoid pigments give three absorption peaks at 425–430, 453–458, and 481–485 nm (Yurkov et al., 1997; Hiraishi et al., 2000a) and together with bacteriochlorophyll *a* produce the colors of orange in young cultures and dark orange-brown in older cultures. In *B. ursincola* bacteriochlorophyll *a* is present in membrane-bound protein-pigment complexes, consisting of the reaction center with absorption peaks at 751, 801, and 853 nm and core LH1 absorbing at 867 nm (Yurkov et al., 1997). The reaction center contains tightly bound tetraheme cytochrome *c*.

Some information is available about the biochemistry of *B. ursincola*. The tricarboxylic acid cycle and the glyoxylate shunt are present, but the key enzyme of the Calvin cycle, ribulose biphosphate carboxylase, is not present (Yurkov et al., 1997). *B. ursincola* is very resistant to tellurite, growing in the presence of 2700 µg/ml in acetate-containing minimal medium (Yurkov et al., 1997). Tellurite is reduced and transformed into metallic tellurium accumulated as metal crystals in the cytoplasm (Yurkov et al., 1996).

Recently, Rickard et al. (2000) reported that strains of *B. natatoria* isolated from biofilms (Buswell et al., 1997) demonstrated intraspecies coaggregation between strains and intergeneric coaggregation with *Micrococcus luteus*. Coaggregation was maximal in the stationary phase of growth, and each member of a coaggregating pair carried either a heat- and protease-sensitive protein (lectin) adhesin or a saccharide receptor, as coaggregation was reversed by sugars.

ENRICHMENT AND ISOLATION PROCEDURES

No enrichment media have been designed for the isolation of *Blastomonas*. The type strain of *B. natatoria* was isolated from a swimming pool using a Millipore *Pseudomonas* Count Water Tester (Sly and Hargreaves, 1984). However, *B. natatoria* may be grown on dilute media such as Staley's peptone yeast glucose medium (Staley, 1981b) on which the colonies appear pink (Sly, 1985). On nutrient agar and R2A agar (Reasoner and Geldreich, 1985) colonies are yellow (Sly, 1985; Rickard et al., 2000).

MAINTENANCE PROCEDURES

Cultures of *Blastomonas* grow well on dilute nutrient media. *B. natatoria* grows well on peptone, yeast extract, glucose (PYG) medium (Staley, 1981b), or R2A medium (Reasoner and Geldreich, 1985). Cultures may be preserved by cryogenic storage in liquid nitrogen when suspended in sucrose peptone broth con-

taining 10% glycerol, and by freeze-drying in glucose peptone broth containing horse serum. Media for *B. ursincola* requires supplementation with 20 µg/l Vitamin B₁₂ (Yurkov et al., 1997).

DIFFERENTIATION OF THE GENUS *BLASTOMONAS* FROM OTHER GENERA

Budding of bacteria is widespread throughout the *Proteobacteria* and occurs in phylogenetically diverse genera with widely different physiological characteristics (Rothe et al., 1987; Hugenholtz et al., 1994; Sly and Cahill, 1997) including *Agromonas*, *Blastobacter*, *Blastomonas*, *Gemmobacter*, *Rhodopseudomonas*, *Methylosinus*, and *Nitrobacter*. Tables BXII.α.90 and BXII.α.91 give the characteristics that are useful in differentiating the species of heterotrophic Gram-negative rod-shaped budding bacteria.

TAXONOMIC COMMENTS

The genus *Blastomonas* was described for the type species *Blastomonas natatoria* (Sly and Cahill, 1997), which was transferred from the genus *Blastobacter* in which it was originally placed (Sly, 1985). Phylogenetic analysis of the members of the genus *Blastobacter* revealed that the genus was polyphyletic and required taxonomic revision (Hugenholtz et al., 1994; Sly and Cahill, 1997). The second species of the genus, *Blastomonas ursincola*, was first described as an aerobic bacteriochlorophyll *a* containing organism and assigned to a new genus *Erythromonas*, as *Erythromonas ursincola*, by Yurkov et al. (1997), even though the type strains of *Blastomonas natatoria* and *Erythromonas ursincola* showed 99.8% 16S rRNA sequence similarity. This taxonomic decision was made with regard to their different physiologies and the important position of photosynthesis in bacterial taxonomy. However later, Hiraishi et al. (2000a) demonstrated that *Blastomonas natatoria* also synthesized bacteriochlorophyll *a* aerobically and contained *puf* genes for photosynthesis. Hiraishi et al. (2000a) consequently transferred *Erythromonas ursincola* to *Blastomonas* and emended the description of the genus to include aerobic bacteriochlorophyll-*a*-synthesizing bacteria.

The taxonomy of *Blastomonas* species continues to be subject to differences of opinion, in particular their relationship to the genus *Sphingomonas* in the family *Sphingomonadaceae*. Takeuchi et al. (2001) proposed that the genus *Sphingomonas* be split into four separate genera (*Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*), primarily on phylogenetic and chemotaxonomic evidence, and proposed that the genera *Blastomonas* and "*Rhizomonas*" be retained to avoid taxonomic confusion. Yabuuchi et al. (1999a), on the other hand, argue that all species in the *Sphingomonadaceae*, including the species of *Blastomonas* be combined into the genus *Sphingomonas*.

Phylogenetically, the genus *Blastomonas* belongs to the *Sphingomonadales*, a monophyletic group in the *Alphaproteobacteria* (Fig. BXII.α.98). *Blastomonas* comprises a monophyletic lineage, not robustly affiliated with any other lineage within the *Sphingomonadales* (including "*Rhizomonas*", which is often shown branching with *Blastomonas*). There is no reproducible (robust) branching order of lineages within the *Sphingomonadales*, so it could be argued that all taxa in the *Sphingomonadales* shown in Fig. BXII.α.98 could be reclassified into the genus *Sphingomonas* to preserve phylogenetic coherence of the group if a "lumping" strategy was adopted. However, it could also be argued that all independent monophyletic lineages within the *Sphingomonadales* (currently there are 10) could be classified as separate genera, thus retaining *Blastomonas* as a distinct genus. Applying this criterion, several species of *Sphingomonas* in Fig. BXII.α.98 would need to be re-

TABLE BXII.α.90. Differential characteristics of the genus *Blastomonas* species and other heterotrophic Gram-negative rod-shaped budding bacteria^a

Characteristic ^b	<i>Blastomonas natalonia</i>	<i>Blastomonas ursincola</i>	<i>Blastobacter henrici</i>	<i>Blastobacter aggregatus</i>	<i>Blastobacter capsulatus</i>	<i>Blastobacter denitrificans</i>	" <i>Blastobacter aminooxidans</i> "	" <i>Blastobacter viscosus</i> "	<i>Agromonas oligotrophica</i>	<i>Gemmibacter aquatilis</i>
<i>Cellular morphology:</i>										
Shape	Rods, slightly curved, or wedge-shaped	Rods, slightly curved, or wedge-shaped	Rods, wedge-shaped, or club-shaped, often curved	Ovoid to rod-shaped	Rods, often bent and tapering on budding pole, older cells Y-shaped	Rods with rounded cell poles	Pleomorphic rods with minute appendages	Pleomorphic rods, often bent and branched	Bent, branched rods	Ovoid to rod-shaped, short chains
Cell length (μm)	1.0–3.0	1.3–2.6	2.0–4.5	1.5–2.3	1.5–2.3	1.5–2.3	1.5–3.0	1.0–3.2	2.0–7.0	1.2–2.7
Cell width (μm)	0.5–0.8	0.8–1.0	0.7–1.0	0.6–0.8	0.7–0.9	0.6–0.8	0.8–1.0	0.5–0.9	0.6–1.0	1.0–1.2
Initial bud shape	Spherical or ovoid	Spherical or ovoid	Spherical or oblong	Rods	Ovoid	Rods	Ovoid	Ovoid	Spherical	Spherical or ovoid
Bud origin	Polar	Polar	Polar	Narrow pole	Narrow pole or lateral	Slightly subpolar	Polar or lateral	Polar or subpolar	Polar	Polar or subpolar
Capsule formation	–	–	ND	–	+	–	+	+	ND	–
Motility	+	+	–	+	–	+	–	–	+	–
Rosette formation	+	–	±	+	–	–	–	–	+	–
Colony pigmentation	Yellow, orange, brown	Yellow orange, brown	ND	Colorless, slightly brownish when older	Colorless	Colorless, brownish when older	Yellow (orange)	Yellow	Colorless	Colorless
Bacteriochlorophyll <i>a</i> produced	+	+	–	–	–	–	–	–	–	–
acrobically										
Origin	Freshwater	Freshwater	Forest brook water	Lake water	Eutrophic pond water	Lake water	Activated sludge	Activated sludge	Paddy soil	Forest pond
Mol% G + C	65	65	ND	60	59	65	69	66	66	63

^aFor symbols see standard definitions.^bData from Doronina et al. (1983), Hirsch and Müller (1985), Hiraishi et al. (2000a), Loginova and Trotsenko (1979), Rothe et al. (1987), Saito et al. (1998), Sly (1985), Sly and Cahill (1997), Trotsenko et al. (1989), Yurkov et al. (1997).

TABLE BXII.α.91. Differential biochemical characteristics of the species of the genus *Blastomonas*^a

Characteristic	<i>Blastomonas natatoria</i>	<i>Blastomonas ursincola</i>
Growth with 3% NaCl ^b	+	—
Hydrolysis of:		
Casein	—	+
DNA	(+)	+
Esculin	+	—
Gelatin	(+)	—
Carbon source utilization: ^c		
L-Arabinose	+	—
D-Glucose	+	(+)
Malate	—	(+)
D-Sorbitol	—	(+)
D-Xylose	++	+

^aData from Hiraishi et al. (2000a).
^bSymbols for physiological and biochemical tests: +, positive; (+), weakly positive; —, negative.
^cSymbols for carbon source utilization tests: ++, good growth; +, moderate growth; —, little or no growth.

classified according to their generic affiliation, and *Erythrobacter*, *Erythromicrobium*, and *Porphyrobacter* may need to be reclassified into a single genus. Papers by Sly and Cahill (1997), Takeuchi et al. (2001), and Yurkov et al. (1997) argue on phylogenetic evidence that *Blastomonas* belongs to a deep separate lineage without a close relationship to the genus *Sphingomonas*. Hiraishi et al. (2000a) proposes that *Blastomonas* should be retained on phylogenetic and phenotypic evidence, particularly its budding morphology and photosynthesis capacity, which clearly separate it from *Sphingomonas*. On the other hand, Yabuuchi et al. (1999a) have proposed the transfer of *B. natatoria* and *B. ursincola* to the genus *Sphingomonas*.

On balance, the authors support the retention of the genus *Blastomonas* at this time to avoid taxonomic confusion. Based on budding morphology and aerobic bacteriochlorophyll *a* synthesis, *Blastomonas* can easily be differentiated from *Sphingomonas*. Hiraishi et al. (2000a) support the retention of the genus *Blastomonas* and note that whereas most species of the genus *Sphingomonas* contain monosaccharide- and oligosaccharide-type glycosphingolipids, *B. natatoria* and *B. ursincola* contain monosaccharide-type glycosphingolipids only.

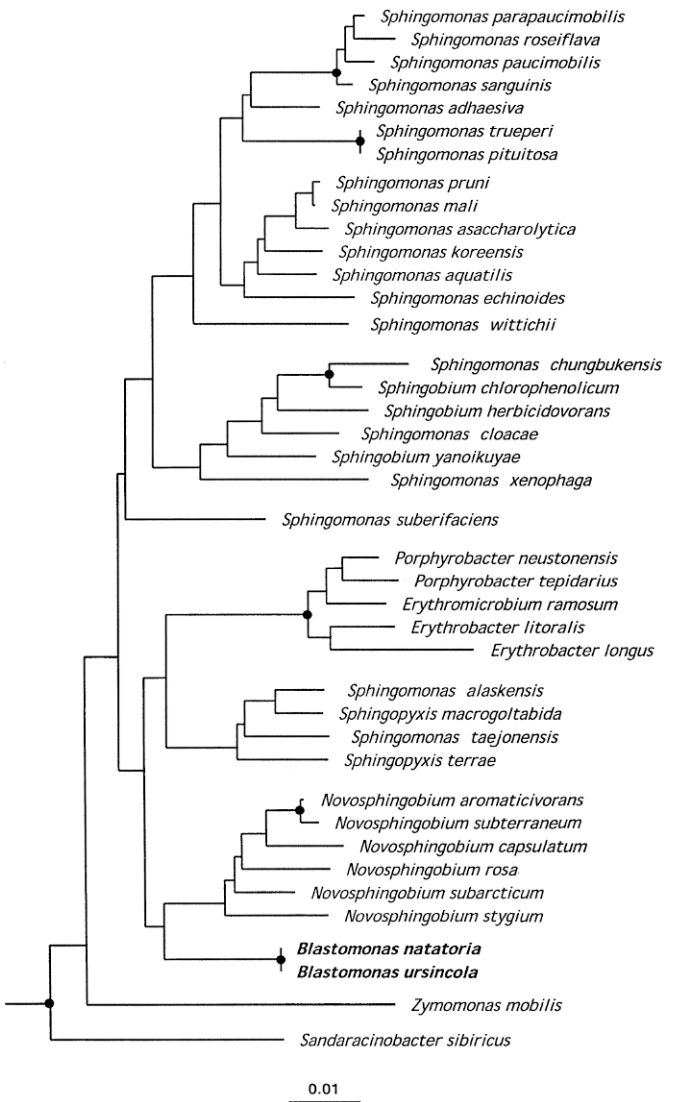


FIGURE BXII.α.98. 16S rRNA evolutionary distance tree of the *Sphingomonadales*. The outgroups used were *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, and *Agrobacterium tumefaciens*. Robust internal nodes (>90 bootstrap confidence) are indicated by a solid circle on the node. The intra-order sequence divergence within the *Sphingomonadales* is 9%.

List of species of the genus Blastomonas

1. *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997, 568^{VP} emend. Hiraishi, Kuraishi and Kawahara 2000a, 1117 (*Blastobacter natatorius* Sly 1985, 43.)
na.ta.to'ri.a. M.L. masc. adj. *natatorius* of a swimming place [pool], the source of the water from which the organism was first isolated.

Cells are ovoid or rod-shaped, 0.6–0.9 × 1–2.5 μm, and reproduce by budding or asymmetric cell division. Colonies on complex media containing peptone and beef extract or yeast extract are circular, convex, smooth, and opaque and grow to 2 mm within 1 week of incubation. Color of colonies is yellow, orange, or brown, depending upon the composition of growth media and age of culture. Aerobic chemoorganotrophy is the preferred mode of growth. No chemolithotrophic growth with H₂, sulfide, or thiosulfate is found. Optimal temperature for growth is 30–35°C. Optimal pH is 7.0–7.5. Growth occurs in the presence of 3% NaCl.

No growth factors are required. Esculin, starch, gelatin, Tween 80, and DNA are hydrolyzed. Chitin, cellulose, and casein are not hydrolyzed. Urease, phenylalanine deaminase, indole, and H₂S are not produced. Good growth occurs with D-xylose, maltose, pyruvate, glutamate, peptone, or yeast extract as sole carbon sources. Other usable carbon sources are L-arabinose, D-glucose, acetate, propionate, butyrate, succinate, fumarate, and Casamino acids. No or little growth occurs with D-fructose, D-mannose, cellobiose, lactose, mannitol, sorbitol, lactate, methanol, propanol, formate, citrate, malate, phenylacetate, benzoate, dichlorophenol, dibenzofuran, dibenzo-*p*-dioxin, or naphthalene. The major phospholipids are phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, and phosphatidylcholine. Inhabits freshwater environments. Isolated from a freshwater swimming pool.

The mol% G + C of the DNA is: 65 (HPLC).

Type strain: ACM 2507, ATCC 35951, DSM 3183, JCM 10396, NCIMB 12085, UQM 2507.

GenBank accession number (16S rRNA): AB024288.

2. **Blastomonas ursincola** (Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997) Hiraishi, Kuraishi and Kawahara 2000a, 1117^{VP} (*Erythromonas ursincola* Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177.)

ur.sin'co.la. M.L. adj. *ursincola* neighbor or compatriot of bears.

Cells are $0.8\text{--}1.0 \times 1.3\text{--}2.6 \mu\text{m}$ in size. No growth occurs in the presence of 3% NaCl. Growth factors are not required, but growth is stimulated significantly by vitamins. Starch, casein, Tween 80, and DNA are hydrolyzed. Esculin,

chitin, cellulose, and gelatin are not hydrolyzed. Urease, phenylalanine deaminase, indole, and H₂S are not produced. Good growth occurs with maltose, pyruvate, glutamate, peptone, or yeast extract as sole carbon source. Other usable carbon sources are D-xylose, D-glucose, D-sorbitol, acetate, propionate, butyrate, succinate, fumarate, malate, and Casamino acids. No or little growth occurs with L-arabinose, D-fructose, D-mannose, cellobiose, lactose, D-mannitol, lactate, methanol, ethanol, propanol, formate, citrate, phenylacetate, benzoate, dichlorophenol, dibenzofuran, dibenzo-*p*-dioxin, or naphthalene. Inhabits freshwater environments. Isolated from a cyanobacterial mat of a thermal spring on Kamchatka Island (Russia).

The mol% G + C of the DNA is: 65 (HPLC).

Type strain: DSM 9006, KR-99.

GenBank accession number (16S rRNA): AB024289.

Genus III. "Citromicrobium" Yurkov, Krieger, Stackebrandt and Beatty 1999, 4523

VLADIMIR V. YURKOV

Ci.tro.mi.cro'bi.um. Gr. n. *citron* citron; Gr. adj. *micro* small; Gr. n. *bios* life; M.L. n. *Citromicrobium* citron-colored microbe.

Cells are pleomorphic, depending on the growth phase of cultures, coccoid to ovoid rods, often forming Y-cells. Motile by one polar or subpolar flagellum. Gram negative, highly variable in its mode of multiplication. Cultures are intensely lemon-yellow colored because of carotenoid pigments, and contain bacteriochlorophyll *a*. Photosynthetic apparatus contains reaction center (RC) and light harvesting (LH) complexes. No growth occurs anaerobically in the light. Incapable of autotrophic growth. Obligately aerobic. No dissimilatory denitrification activity detected. The habitat is marine.

The mol% G + C of the DNA is: 67.5.

Type species: "**Citromicrobium bathyomarinum**" Yurkov, Krieger, Stackebrandt and Beatty 1999, 4523.

FURTHER DESCRIPTIVE INFORMATION

"*Citromicrobium bathyomarinum*" is the only "*Citromicrobium*" species currently described. Phylogenetically, this species is closely related to other aerobic phototrophic bacteria and belongs to the *Alphaproteobacteria*.

On agar plates, "*C. bathyomarinum*" forms small (2–4 mm) lemon-yellow colonies with a smooth surface. With age, the color of the colonies turns intensely yellow. After growth aerobically in liquid media aerated by shaking, cultures are slightly aggregative, and bright yellow. The culture does not grow anaerobically in the light or under dark conditions. In agar (0.7%) deeps, growth occurs only at the surface (the aerobic and semiaerobic zones) of agar tubes. Light is not required for growth.

Cells of strain "*C. bathyomarinum*" grown in rich organic (RO) liquid medium that contains 0, 0.5, 1.0, 2.0, 3.0, or 5.0% NaCl, are similar morphologically and unusually pleomorphic. Depending on the growth phase of cultures, the morphology of cells ranges from almost coccoid ($0.4\text{--}0.5 \times 0.5\text{--}0.8 \mu\text{m}$), through ovoid rods ($0.4\text{--}0.5 \times 1.0\text{--}1.2 \mu\text{m}$), to thread-like formations of up to 5 cells. In RO medium with higher NaCl content (7% or 10%), bean shaped or wavy cells as well as long thread-like formations of up to 10 cells are found. Coccoid cells from young cultures are motile by one polar or subpolar flagellum (Yurkov and Beatty, 1998b; Yurkov et al., 1999).

"*C. bathyomarinum*" is variable in its mode of cell division.

Budding, ternary fission, binary division, and symmetric and asymmetric constrictions were observed. This bacterium forms Y-cells, a rare type of bacterial multiplication, which results in the possibility of three daughter cells produced from one mother cell (Fig. BXII.α.99). Cells often remain attached after division, apparently by means of a membranous connective material of unknown nature, and are surrounded by a thin "bubbly" substance (Fig. BXII.α.99). Therefore, cells in liquid culture are slightly aggregative, such that many individual cells remain in contact after division.

Electron microscopic thin sections showed that "*C. bathyomarinum*" has a Gram-negative cell wall. The cytoplasmic membrane was visible, but no obvious intracytoplasmic membranes (ICMs) were detected. No inclusions indicative of storage materials were seen in cells harvested from RO medium.

The *in vivo* absorption spectrum of "*C. bathyomarinum*" has a major peak at 867 nm, indicating the presence of bacteriochlorophyll *a* incorporated into LH complex I (LHI). The small peak at 800 nm indicates the presence of the photosynthetic RC. The photosynthetic apparatus organization of an LH system associated with the RC (photosynthetic unit) in this species was indicated by isolation and purification of LHI-RC particles after lysis and treatment of cells with detergent, and sucrose gradient fractionation. In addition to the LHI-RC-enriched fraction, another fraction that contained a low amount of apparently an LHII complex (absorption peaks at 799 and 849 nm) was reported (Yurkov et al., 1999).

The above data indicate the presence of LHI, LHII, and RC complexes in cells of "*C. bathyomarinum*", and the total number of photosynthetic units per cell is similar to the number of units determined from the spectra of other aerobic phototrophic bacteria. Cells contain 0.4–0.6 nmol of BChl/mg of protein. "*C. bathyomarinum*" produces BChl when grown aerobically in the dark, but production of this pigment is significantly repressed on rich media such as RO, or media containing yeast extract or Casamino acids. The most pronounced BChl synthesis was detected in a minimal medium containing acetate, glutamate, or butyrate as the sole source of carbon.

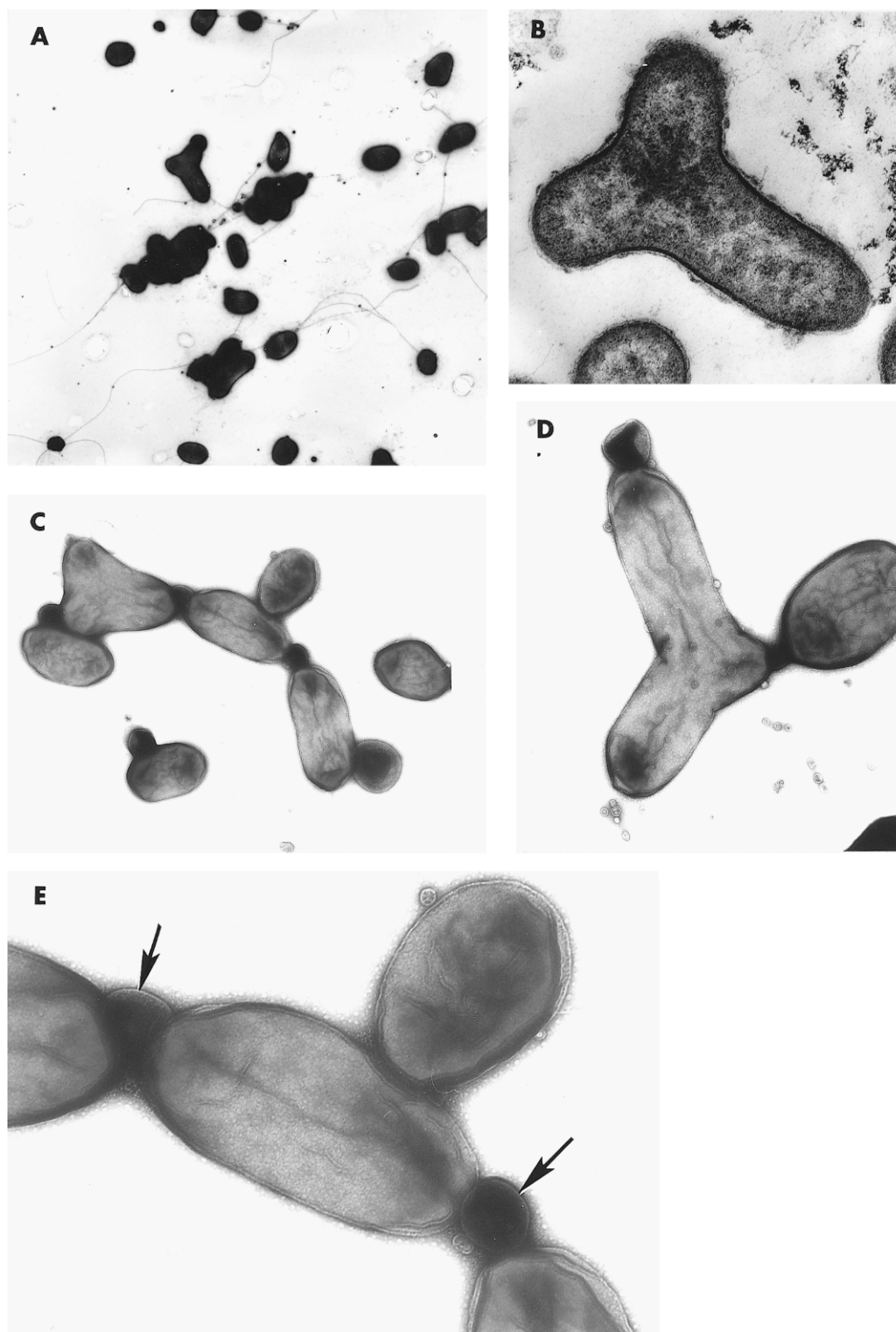


FIGURE BXII.α.99. Electron micrographs of negatively stained (A, C, D, E) and thin-sectioned (B) cells of "*Citromicrobium bathyomarinum*". (A) Pleomorphism of cells, (B) Y-shaped form during early stage of cell division. The nucleoid is distributed in three directions, (C and D) Cells of different morphologies connected by an unknown material, (E) Enlarged fragment of panel C, showing membranous connective material (arrows).

The yellow color of cells and the three peaks at 433, 457, and 487 nm indicate the presence of carotenoids, apparently of the carotene type. The ratio of the absorbance at the LHI BChl absorption peak (867 nm) to that at the main carotenoid absorption peak (457 nm) is about 1:8. However, the ratio of carotenoids:BChl was much higher in intact cells than in partially purified photosynthetic complexes. It seems that most of these carotenoids were not intimately bound to the components of the photosynthetic apparatus (LH and RC). As noted above, ICM invaginations were not observed in the electron microscopic thin sections. Possibly the photosynthetic apparatus of "*C. bathyomarinum*" is restricted to the cytoplasmic membrane.

"*C. bathyomarinum*" is broadly tolerant to culture conditions such as salinity, temperature, and pH. Thus, growth occurs in a

freshwater medium and a medium supplemented with 10% NaCl, at temperatures ranging from 4° to 45°C, and at pH values of 5.5–10.0. Therefore, "*C. bathyomarinum*" is a salt-, pH-, and thermotolerant strain.

"*C. bathyomarinum*" utilizes an unusually low number of substrates compared to other aerobic phototrophic bacteria. Glutamate, butyrate, and yeast extract are the best carbon sources, and acetate and glucose support weak growth. The substitution of nitrate for ammonia as a nitrogen source, and an increase or decrease in aeration did not affect the variety of organic substrates that supported growth. Gelatin is not hydrolyzed, but Tween 20, Tween 80, and starch are hydrolyzed, indicating the absence of gelatinase and the presence of lipolytic and amylolytic activities (Table BXII.α.92).

TABLE BXII.α.92. Growth and physiological properties of "*Citromicrobium bathyomarinum*" strain JF-1 and phylogenetically related species^a

Characteristic	" <i>Citromicrobium bathyomarinum</i> " JF-1	<i>Blastomonas ursincola</i>	<i>Erythrobacter longus</i>	<i>Erythromicrobium ramosum</i>	<i>Porphyrobacter neustonensis</i>
<i>Growth at pH:</i>					
5	—	—	—	—	—
5.5	+	—	—	—	—
6	+	+	—	+	—
7	+	+	+	+	+
8	+	+	+	+	+
9	+	+	+	+	+
9.5	+	+	+	+	—
10	w	—	—	—	—
<i>Growth at (°C):</i>					
4	+	—	w	—	—
15	+	+	+	+	w
20	+	+	+	+	+
30	+	+	+	+	+
37	+	w	w	—	w
40	+	—	—	—	—
42	+	—	—	—	—
45	+	—	—	—	—
50	—	—	—	—	—
<i>Utilization of:</i>					
Acetate	w	+	—	+	—
Pyruvate	—	+	+	+	+
L-Glutamate	+	+	+	+	—
Butyrate	+	+	+	+	—
Citrate	—	+	+	+	—
Malate	—	+	—	+	—
Succinate	—	+	—	+	+
Lactate	—	+	nd	+	—
Formate	—	—	nd	—	—
D-Glucose	w	+	+	+	+
D-Fructose	—	+	+	+	+
Methanol	—	—	—	—	—
Ethanol	—	—	nd	+	—
Yeast extract	+	+	+	+	+
<i>Hydrolysis of:</i>					
Gelatin	—	—	+	—	—
Tween 20	+	+	nd	—	+
Tween 80	+	+	+	—	+
Starch	+	—	—	—	—
<i>Reduction of:</i>					
NO ₃ [−] to NO ₂ [−]	—	—	+	—	nd
NO ₂ [−] to N ₂	—	—	—	—	—
TeO ₃ ^{−2} to Te	+	+	+	+	nd
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
<i>Susceptibility to:</i>					
Chloramphenicol (100 µg)	+	+	+	+	+
Penicillin (20 µg)	—	—	—	—	+
Streptomycin (50 µg)	—	—	—	—	—
Fusidic acid (0.5 µg)	+	+	nd	+	nd
Polymixin B (100 µg)	+	+	—	+	nd

^aFor symbols see standard definitions; nd, not determined; w, weak growth.

As a good representative of the aerobic phototrophic bacteria, "*C. bathyomarinum*" is highly resistant to tellurite and reduces this compound to tellurium apparent as intracellular crystals with the minimal inhibitory concentration of tellurite shown to be 2000 µg/ml (Yurkov et al., 1999).

ENRICHMENT AND ISOLATION PROCEDURES

"*C. bathyomarinum*" strain JF-1 was isolated from samples obtained from the vicinity of nonbuoyant regions of plumes emitted from hydrothermal vents on the Juan de Fuca Ridge (Northeastern Pacific Ocean; ~47°57'N, 129°05'W; about 2000 m beneath the ocean surface). Descriptions of the samples and bacterial heterotrophic population enumerated on the medium used are given in Yurkov and Beatty (1998b). The techniques used to isolate and cultivate "*C. bathyomarinum*" are similar to those described for *Erythromicrobium*.

MAINTENANCE PROCEDURES

"*Citromicrobium*" can be preserved by standard procedures in liquid nitrogen, by freezing at -70°C or below, or lyophilization (see chapter on *Erythromicrobium* for details).

DIFFERENTIATION OF THE GENUS *CITROMICROBIUM* FROM OTHER GENERA

Genus "*Citromicrobium*" can be easily differentiated from other known genera of the aerobic phototrophic bacteria. On a phylogenetic tree based on 16S rDNA sequences, "*C. bathyomarinum*" represents an independent branch within the *Alphaproteobacteria*. This organism has a very characteristic lemon yellow color, which has been used to compose a taxonomic name for this species. "*C. bathyomarinum*" is very pleomorphic (Fig. BXII.α.99) and variable in its mode of reproduction. The Y-cell type of cellular division, a type rarely observed among bacteria, has been found as one of the ways "*C. bathyomarinum*" reproduces itself (Fig. BXII.α.99). Cells often remain attached after division, apparently by means of membranous connective material of unknown nature. This species is capable of growth over a broad range of NaCl concentrations, pH values, and temperatures. However, it

seems to have quite limited metabolic possibilities, being able to grow on an unusually low (for this physiological group) number of substrates (Table BXII.α.92).

TAXONOMIC COMMENTS

The 16S rDNA sequences of strain JF-1 showed that the phylogenetically closest relatives of the genus "*Citromicrobium*" were members of the genera *Sphingomonas*, *Sandaracinobacter*, *Erythrobacter*, *Erythromonas* (*Sphingomonas*), and *Porphyrobacter* (Fig. BXII.α.100). The phylogenetic position of "*C. bathyomarinum*" is as a branch between the genus *Erythromonas* (*Sphingomonas*) and the *Erythromicrobium*-*Porphyrobacter*-*Erythrobacter* cluster within the *Alphaproteobacteria*.

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105-122.
Yurkov, V.V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695-724.

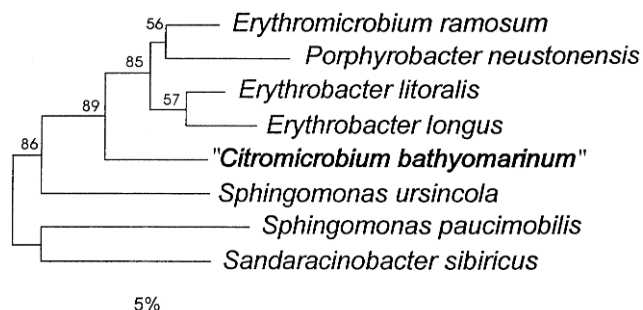


FIGURE BXII.α.100. Dendrogram showing the phylogenetic position of "*Citromicrobium bathyomarinum*" among members of the genera *Sphingomonas*, *Erythrobacter*, *Sandaracinobacter*, and *Porphyrobacter*. Note: *Blastomonas ursincola* (Yurkov et al. 1997) Hiraishi et al. 2000a, 1117 is a junior objective synonym of *Sphingomonas ursincola* (Yabuuchi et al., 2002). Bar = 5% sequence divergence.

List of species of the genus "*Citromicrobium*"

1. "*Citromicrobium bathyomarinum*" Yurkov, Krieger, Stackebrandt and Beatty 1999, 4524.

ba.thy.o.ma.ri' num. Gr. adj. *bathys* deep; L. adj. *marinum* oceanic; M.L. adj. *bathyomarinum* deeply oceanic.

Gram negative, lemon-yellow pigmented, pleomorphic. Cells may be almost coccoid ($0.4\text{--}0.5 \times 0.5\text{--}0.8 \mu\text{m}$), ovoid rods ($0.4\text{--}0.5 \times 1.0\text{--}1.2 \mu\text{m}$), or thread-like structures. Coccoid cells are motile by means of one polar or subpolar flagellum. Cells contain BChl *a* and carotenoid pigments. BChl *a* bound to proteins gives *in vivo* absorption peaks at 800 and 867 nm. Photosynthetic apparatus is organized in RC, LHI, and LHII complexes, as evidenced by partially purified preparations in which the LHI complex yields a peak at 866 nm and the LHII complex at 799 and 849 nm.

Aerobic, chemoorganotroph, and facultative photoheterotroph. The best growth substrates are L-glutamate, butyrate, and yeast extract; weak growth on minimal media containing acetate or D-glucose. No growth on pyruvate, citrate, malate, succinate, lactate, formate, D-fructose, meth-

anol, or ethanol. Optimal temperature for growth 20–42°C. Capable of growth over a salinity range from 0% to 10% NaCl in RO medium with an optimum of 1–5%. The pH optimum is from 6.0 to 8.0. Exhibits oxidase, catalase, lipase, and amylase, but not gelatinase activities. No dissimilatory denitrification, or anaerobic growth in the presence of trimethylamine oxide (TMAO). Glucose is fermented to acid products without gas generation. Demonstrates a high level of resistance to tellurite (up to 2000 µg/ml in an acetate-glutamate minimal medium). Tellurite is reduced and transformed to metallic tellurium causing blackening of the culture. Resistant to penicillin and streptomycin; sensitive to chloramphenicol, fusidic acid, and polymyxin B.

Habitat: the vicinity of nonbuoyant regions of plumes emitted from hydrothermal vents on the Juan de Fuca Ridge (northeastern Pacific Ocean).

The mol% G + C of the DNA is: 67.5 (HPLC).

Deposited strain: JF-1.

GenBank accession number (16S rRNA): Y16267.

Genus IV. Erythrobacter Shiba and Simidu 1982, 215^{VP}

TSUNEO SHIBA AND JOHANNES F. IMHOFF

E.ryth'ro.bac.ter. Gr. adj. *erythros* red; M.L. masc. n. *bacter* rod; M.L. masc. n. *Erythrobacter* red rod.

Cells are ovoid to rod-shaped, $0.2\text{--}0.4 \times 1.0\text{--}5.0 \mu\text{m}$, multiply by binary fission, and are motile by means of polar or subpolar flagella. Gram negative and belong to the *Alphaproteobacteria*. Cell suspensions and colonies are orange or pink. **Bacteriochlorophyll *a*** is present. **The major respiratory quinone is ubiquinone-10.** The main cellular fatty acid is $\text{C}_{18:1}$.

Aerobic chemoorganoheterotrophic bacteria. Do not grow chemoautotrophically or under anoxic conditions in the light. Metabolism is predominantly respiratory. Under microoxic conditions, small amounts of acid are produced from carbohydrates. Oxidase and catalase positive. Tween 80 is hydrolyzed.

Mesophilic and neutrophilic bacteria from the marine environment. Optimum growth is at pH 7.0–8.0 and 25–30°C. Grow well at salt concentrations of 0.1–9.6%.

The mol% *G + C* of the DNA is: 57–67.

Type species: ***Erythrobacter longus* Shiba and Simidu 1982, 216.**

FURTHER DESCRIPTIVE INFORMATION

Erythrobacter belongs to the *Alphaproteobacteria*, together with *Porphyrobacter*, *Erythromicrobium*, "*Citromicrobium*," *Erythromonas*, *Sandarinobacter*, *Sphingomonas*, and *Blastomonas*. Although *Erythrobacter* contains bacteriochlorophyll *a* (Bchl *a*), internal membrane systems are not present. The cellular level of Bchl is only around 2 nmol/mg of dry weight for *E. longus*, or 0.9–3.9 nmol/mg of protein for *E. litoralis*, and comparable to the level in heterotrophically grown cells of most purple nonsulfur bacteria (Harashima et al., 1978; Yurkov et al., 1994c). A reaction center–light-harvesting complex I (RC-LH I) is present, but not a light-harvesting complex II. In the near infrared region, the *in vivo* absorption spectrum of Bchl *a* reveals a minor maximum at 800–807 nm and a major one at 866–868 nm. *Erythrobacter* contains a large amount of polar carotenoids not included in the RC-LH I complex (Shimada et al., 1985) and not engaged in photosynthetic activities (Noguchi et al., 1992). Among about 20 different polar carotenoids, the most abundant are erythroanthin sulfate and caloxanthin sulfate (Takaichi et al., 1991). The RC-LH I complex contains bacteriorubixanthin, zeaxanthin, and hydroxyderivatives of β -carotene (Takaichi et al., 1988). Terminal oxidase in the respiratory system of *E. longus* is *aa₃*-type cytochrome oxidase (Fukumori et al., 1987).

The amine composition of *E. longus* is characteristic, in that only spermidine, but no appreciable amounts of putrescine, is found (Hamana et al., 1985). A high level of melatonin, *N*-acetyl-5-methoxytryptamine, which is known to offer protection against oxygen radicals, is found in *E. longus*. Cell homogenates of dark-grown, but not of light-grown, cells retain melatonin-producing enzyme activity (Tilden et al., 1997).

Erythrobacter grows as an aerobic chemoorganoheterotroph (Shiba and Simidu, 1982). Neither phototrophic growth nor light-dependent ATP synthesis are found, though an operative light-driven cyclic electron transfer system occurs (Harashima et al., 1982). The physiological function of Bchl *a* is not yet known. In contrast to anoxygenic phototrophic bacteria, Bchl synthesis is inhibited by light, but not suppressed at atmospheric oxygen tension (Harashima et al., 1980, 1987; Shiba, 1987). *E. litoralis* is quite resistant to tellurite (Yurkov et al., 1996).

E. litoralis was isolated from a cyanobacterial mat in a supralittoral zone of the North Sea, which is flooded approximately

twice a month. Due to rainfall and evaporation induced by wind and sunlight, the salinity in the pore water may fluctuate greatly and reach high levels. Growth of *E. litoralis* at salt concentrations of 0.5–9.6% may reflect adaptation to these conditions (Yurkov et al., 1994c).

E. longus was isolated from the green seaweed *Enteromorpha linza* distributed in a high intertidal zone. This species can also grow in a wide salinity range of 0.1–7% (Sato et al., 1989; Shiba et al., 1991; Shiba, 1995).

ENRICHMENT AND ISOLATION PROCEDURES

Enrichment of *Erythrobacter* species has been achieved from marine beach sand, surface seawater, and several seaweeds. Samples were diluted 10-fold with seawater and spread on agar plates. The agar plates were incubated at 20°C. Characteristic pink or orange colonies were streaked out on further agar plates until pure cultures were obtained.

The culture medium used for *E. litoralis* contained per liter of distilled water: 1.0 g sodium acetate, 1.0 g yeast extract (Difco), 1.0 g Bacto Peptone (Difco), 15.0 g NaCl, 0.3 g KCl, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g NH_4Cl , 0.3 g K_2HPO_4 , 20 μg vitamin B_{12} , and 1 ml of a trace element solution (Drews, 1983). The pH is adjusted to 7.6–7.8.

For *E. longus*, the modified PPES-II medium (Shiba, 1989) was used, containing per liter: 2 g polypeptone (Nihon Pharmaceutical Co.), 1 g proteose–peptone (Difco no. 3), 1 g Bacto soytone (Difco), 1 g Bacto yeast extract (Difco), 0.1 g ferric citrate, 300 ml of distilled water, 700 ml artificial seawater (Lyman and Fleming, 1940), and 15 g agar.

MAINTENANCE PROCEDURES

Erythrobacter can be maintained at 20°C as stab cultures in media containing 0.4% agar (Difco). Cultures should be transferred monthly. Maintenance on agar slant cultures at room temperature is not recommended. The bacteria remain viable for 6 months when slant cultures are kept at -80°C . Cultures may also be preserved in liquid nitrogen or by lyophilization.

DIFFERENTIATION OF THE GENUS *ERYTHROBACTER* FROM OTHER GENERA

Erythrobacter differs from the genus *Sphingomonas* by the presence of Bchl *a* (Takeuchi et al., 1994). The color of cell suspensions and colonies of *Erythrobacter* is orange or pink, while those of *Sphingomonas* are yellow or white. The difference in colony color suggests differences in carotenoid composition. *Erythrobacter* also differs from the two genera in the presence of 3-hydroxy fatty acids (Urakami and Komagata, 1988; Takeuchi et al., 1993a).

Erythrobacter can be distinguished from *Erythromicrobium* by the composition of Bchl-protein complexes. *Erythrobacter* contains only a RC-LH I complex, while *Erythromicrobium* contains RC-LH I and LH II complexes. In addition to near infrared absorption maxima at 799 and 870 nm, *Erythromicrobium* has a maximum at 838 nm (Shimada et al., 1985; Yurkov et al., 1997).

Cell form and type of fission distinguish *Erythrobacter* from *Blastomonas*. *Erythrobacter* is rod shaped and reproduces by binary fission, while *Blastomonas* is ovoid and reproduces by budding. It does not contain 3-hydroxy fatty acids. *Erythrobacter* and *Sandarinobacter* are distinguished by their color: colonies of *Erythro-*

bacter are orange or pink and show an *in vivo* absorption maximum at 470 nm, while those of *Sandaracinobacter* are yellow-orange and have maxima at 424, 450, and 474 nm (Shiba and Simidu, 1982; Yurkov et al., 1997).

Erythrobacter differ from *Porphyrobacter* in carotenoid composition. *Erythrobacter* contain erythroanthin sulfate as a major polar carotenoid, while *Porphyrobacter* contain a carotenoid sulfate, the structure of which is not yet identified, but different from erythroanthin sulfate. Zeaxanthin is a major non-polar carotenoid in *Erythrobacter*, whereas it is minor in *Porphyrobacter* (Takachi et al., 1990 1991; Hanada et al., 1997). *Erythrobacter* species were isolated from marine environments and show a slight requirement for sodium ion, while *Porphyrobacter* species were isolated from terrestrial environments and do not have a

requirement for sodium (Shiba and Simidu, 1982; Yurkov et al., 1994c). *Erythrobacter* are different from "*Citromicrobium*" in colony color and cell form. "*Citromicrobium*" is yellow-pigmented and pleomorphic.

TAXONOMIC COMMENTS

The genus *Erythrobacter* contains two species. The strain OCh114, assigned to the genus *Erythrobacter*, was originally transferred to the genus *Roseobacter* as *Roseobacter denitrificans* (Shiba, 1991a). Based on 16S rDNA sequence analysis, "*Erythrobacter sibiricus*" has been removed from the genus (Yurkov and Gorlenko, 1990). After tentative assignment to the genus *Erythromicrobium*, this species is now included in a new genus as *Sandaracinobacter sibiricus* (Yurkov et al., 1997).

List of species of the genus *Erythrobacter*

1. *Erythrobacter longus* Shiba and Simidu 1982, 216^{VP}

lon'gus. L. adj. *longus* long.

Cells are long rods, 0.3–0.4 × 1.0–5.0 µm, motile by means of subpolar flagella. The cells do not form internal membranes. Cell suspensions and colonies are orange, having *in vivo* absorption maxima at 807 (minor) and at 470 nm and 866–867 nm (major). Bacteriochlorophyll *a* esterified with phytol is present. Contain erythroanthin sulfate and bacteriorubixanthinal as major carotenoids. Main cellular fatty acid is C_{18:1}. C_{15:0} 2OH and C_{14:0} 2OH are present.

Aerobic, chemoorganoheterotrophic, predominantly respiratory metabolism. Utilize glucose, acetate, pyruvate, glutamate, and butyrate as sole carbon sources. Methanol is not utilized. Some strains reduce nitrate to nitrite. Oxidase, catalase, and phosphatase positive. The Voges–Proskauer and methyl-red tests are negative. H₂S is not produced. Indole is produced. Tween 80 and gelatin are hydrolyzed. Some strains hydrolyze alginate. Biotin is required as growth factor.

Mesophilic and neutrophilic bacterium with optimal growth at pH 7.0–8.0 and 25–30°C. Good growth at salt concentrations of 0.1–7.0%.

Habitat: oxic marine environments, especially on seaweeds.

The mol% G + C of the DNA is: 57 (HPLC).

Type strain: OCh101, ATCC 33941, DSM 6997, IFO 14126.

GenBank accession number (16S rRNA): L01786, M96744, M59062.

2. *Erythrobacter litoralis* Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 432^{VP}

li.to.ra'lis. L. gen. n. *litoris* of the seashore; M.L. masc. adj. *litoralis* belonging or pertaining to the seashore.

Cells are rod shaped, 0.2–0.3 × 1.0–1.3 µm, motile by means of polar or subpolar flagella. Short chains of up to five cells may occur. Internal membranes are not formed. Color of cell suspensions and colonies is pink or orange, but become red or brown in older cultures. Near-infrared absorption maxima are at 800 and 868 nm. Bchl *a* is present. The main carotenoids are bacteriorubixanthinal and erythroanthin sulfate.

Aerobic chemoorganoheterotrophic metabolism. Growth occurs on glucose, fructose, butyrate, glutamate, acetate, and lactate, and weak growth on succinate. Methanol is not utilized. Nitrate is not reduced. Tween 80 is hydrolyzed; gelatin and starch are not. Catalase and oxidase positive. Susceptible to chloramphenicol, tetracycline, and fusidic acid. Resistant to penicillin, streptomycin, and polymyxin B.

Mesophilic and neutrophilic bacterium with optimal growth at pH 7.0–8.0 and 25–30°C. Good growth at salt concentrations of 0.5–9.6%.

Habitat: marine cyanobacterial mat in supralittoral zones.

The mol% G + C of the DNA is: 67 (T_m).

Type strain: T4, DSM 8509, IAM 14332.

GenBank accession number (16S rRNA): ABO13354.

Genus V. *Erythromicrobium* Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 432^{VP}

VLADIMIR V. YURKOV

E.ry'thro.mi.cro'bi.um. Gr. adj. *erythrus* red; Gr. adj. *micros* small; Gr. n. *bios* life; M.L. n. *Erythromicrobium* red microbe.

Pleomorphic bacteria, Gram negative, and usually motile by means of flagella. Divide by binary or ternary fission. **Real branching may occur**. Colonies are red-orange. Cells **contain bacteriochlorophyll *a* and carotenoids**.

Aerobic. Cultures do not grow anaerobically in the light and do not grow chemoautotrophically. Ribulose diphosphate carboxylase is not detected. No fermentation and no denitrification activities occur.

The mol% G + C of the DNA is: 62.5–68.5.

Type species: *Erythromicrobium ramosum* Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 432.

FURTHER DESCRIPTIVE INFORMATION

Phylogenetically, the genus *Erythromicrobium* is associated with members of the *Alphaproteobacteria* and is closely related to *Ery-*



FIGURE BXII.α.101. *Erythromicrobium ramosum*. Ramifying thread-like cell showing true branching. Electron micrograph, ultrathin section. Bar = 0.5 μm. (Printed with permission from V. Yurkov.)

throbacter (level of 16S rDNA sequence similarity, 98%) (Yurkov et al., 1994c). The genera *Erythromicrobium*, *Erythrobacter*, and *Porphyrobacter* form a phylogenetic cluster in the *Alphaproteobacteria*. The genus *Sphingomonas* was shown to be the most closely related nonphotosynthetic genus within the *Alphaproteobacteria* (Yurkov et al., 1994c).

Three described species of *Erythromicrobium*—*E. ramosum*, “*E. ezovicum*”, and “*E. hydrolyticum*”—are very long rods and produce characteristic thread-like cells, dividing by symmetric or asymmetric constriction. For *E. ramosum* and “*E. hydrolyticum*” ternary fission and branching were demonstrated (Fig. BXII.α.101) (Yurkov and Gorlenko, 1992).

All *Erythromicrobium* species synthesize a large amount of carotenoid pigments that determine the color of the organism. *E. ramosum* was shown to produce at least 10 different kinds of carotenoids (Yurkov et al., 1993). The two predominant carotenoids, the orange erythroxanthin sulfate and the red bacteriorubixanthin, are very polar. The *in vivo* absorption spectra of “*E. ezovicum*”, “*E. hydrolyticum*”, and *E. ramosum* all have major carotenoid peaks at 466 and 478 nm, indicating a similar carotenoid composition. The carotenoids impact an intense color in liquid cultures, red-orange (Yurkov et al., 1997).

The photosynthetic apparatus of *Erythromicrobium* contains a reaction center and two types of antenna complexes, LHI and LHII. Reaction centers do not possess reaction center-bound cytochrome *c*, and the photooxidized special pair (P^+) of the reaction center is directly reduced by a soluble cytochrome *c* (Yurkov et al., 1995, 1998a). LHI with absorption maximum at 871 nm is similar to that measured in many anaerobic phototrophic and other aerobic phototrophic bacteria. Isolated LHII showed bacteriochlorophyll absorption maxima at 798–800 and 832–833 nm, indicating the presence of new types of LHII in *Erythromicrobium* cells. The long-wavelength band of LHII at 832–

833 nm is about 20 nm less than that usually observed in purple bacteria (Yurkov et al., 1997). Although it has some peculiarities, the photosynthetic apparatus of *Erythromicrobium* is functional in terms of a cyclic electron transfer system. The photoinduced cyclic electron transfer occurs only under relatively oxidized (aerobic) conditions, as elucidated by light-induced absorbance changes in intact cells. Under anaerobic conditions, no light-induced reaction center absorbance changes were observed. The lack of photochemistry under anaerobic conditions is consistent with the inability of these bacteria to grow by light-dependent photophosphorylation in the absence of oxygen (Yurkov et al., 1995, 1998a).

Erythromicrobium species possess ubiquinone Q-10 as the major quinone. No menaquinones or rhodoquinones have been detected. The ubiquinone Q-9 was detected as a minor quinone in addition to Q-10 in *E. ramosum*, “*E. ezovicum*”, and “*E. hydrolyticum*”. The quinone Q-10 of “*E. hydrolyticum*” and *E. ramosum* seems to exist as a methylated form (Gogotov and Gorlenko, 1995).

The genus *Erythromicrobium* demonstrated high-level resistance to tellurite and accumulation of metallic tellurium crystals due to tellurite reduction (Fig. BXII.α.102) (Yurkov et al., 1996). Tellurite resistance and tellurium accumulation depend on medium composition, particularly on organic carbon source (Table BXII.α.93).

The major determinative characteristics of the genus *Erythromicrobium* are summarized in Table BXII.α.94.

ENRICHMENT AND ISOLATION PROCEDURES

Erythromicrobium species have been isolated from the samples of freshwater cyanobacterial mats that developed in alkaline warm springs (pH 9.5, 25°C) of the Bol'shaya River valley (Baykal Lake region in Russia). It was isolated by direct inoculation of a ho-

mogenized mat sample with dilutions on agar plates of rich organic (RO) medium (Yurkov et al., 1994c) containing (g/l): yeast extract, 1.0; Bacto peptone, 1.0; sodium acetate, 1.0; KCl, 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05; NH_4Cl , 0.3; K_2HPO_4 , 0.3. The medium was supplemented with 20 mg/l of vitamin B_{12} and 1.0 ml/l of a trace element solution (Drews, 1983). The plates were cultivated in the dark at 30°C and pH 7.6–7.8. Characteristic red-orange colonies are streaked out on agar plates by the usual aerobic techniques. When a pure culture is obtained, a single colony is transferred into liquid RO medium and cultivated in an Erlenmeyer flask aerobically in the dark.

MAINTENANCE PROCEDURES

Erythromicrobium liquid cultures (taken from late logarithmic growth phase) and agar surface cultures remained viable after storage at 4°C for at least 2 months. Long-term preservation of *Erythromicrobium* species is possible by storage in liquid nitrogen or freezing at -70°C . For this purpose, heavy cell suspensions of liquid cultures (mid-logarithmic growth phase) are supplemented with glycerol (final concentration, 30%) as a protective agent. These cell suspensions are filled into 2-ml plastic screw-cap tubes and freeze-stored. Lyophilization can also be used as a method for *Erythromicrobium* preservation.

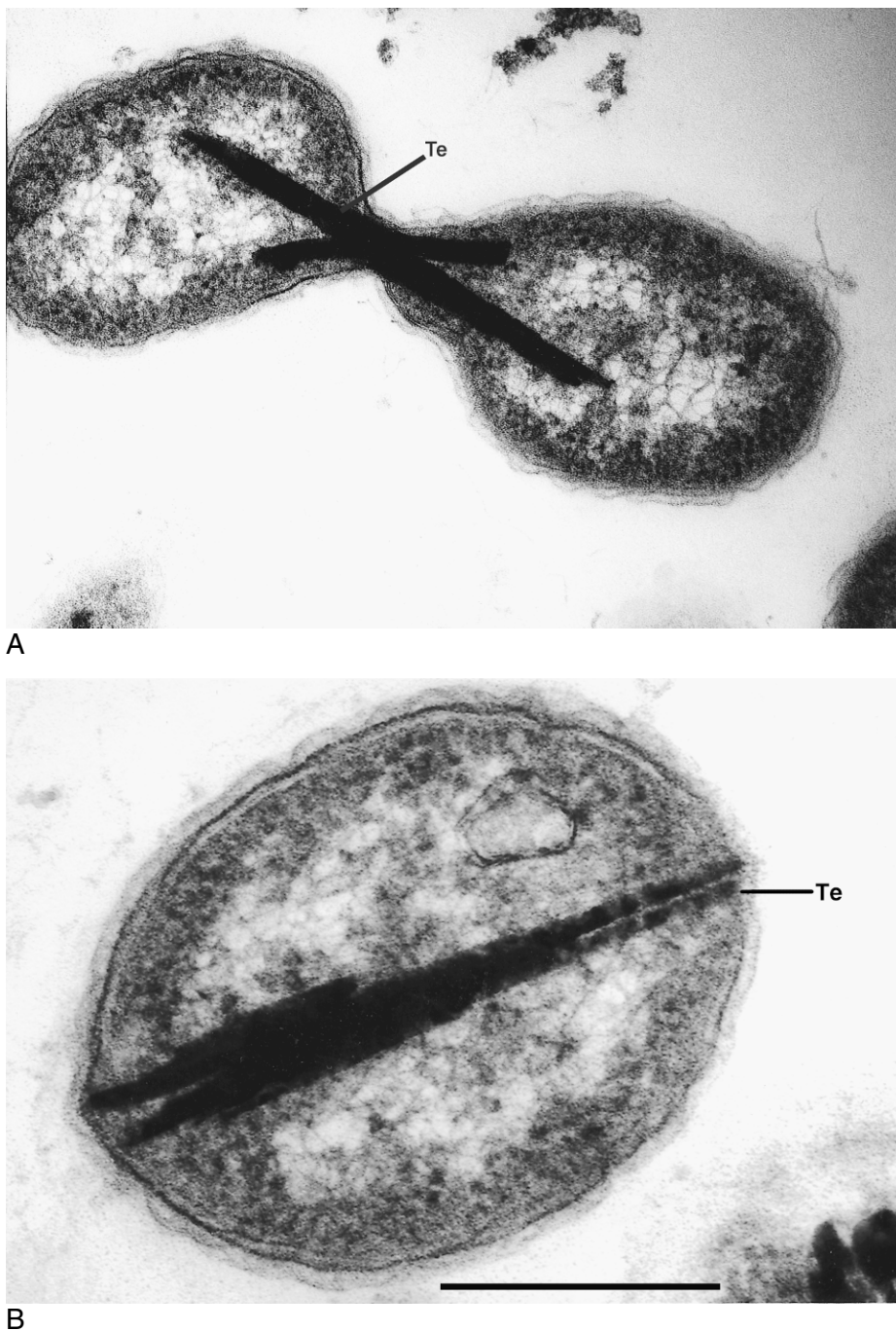


FIGURE BXII.α.102. Intracellular localization of accumulated tellurium (Te ; indicated by arrows) by *Erythromicrobium ramosum*. (Printed with permission from V. Yurkov.) A, Two daughter cells with tellurium crystals apparently interfering with cell division. B, Long tellurium crystals extending across cell. Bars = 0.25 μm.

TABLE BXII.α.93. Reduction of K_2TeO_3 by *Erythromicrobium* species depending on organic carbon source^a

Carbon source	<i>E. ramosum</i> ^b			<i>"E. ezovicum"</i> ^b			<i>"E. hydrolyticum"</i> ^b		
	A	B	C	A	B	C	A	B	C
R.O. ^c	+	50	750	+	500	1200	+	n.r.	5
Yeast extract	+	250	1500	+	500	2000	+	n.r.	5
L-Glutamine	+	250	1200	+	750	1200	+	2000	2000
Succinate	+	500	1200	+	750	1200	+	2000	2000
Malate	+	250	1200	+	750	1200	+	1200	1200
Tartrate	±	100	500	±	n.r.	5	±	5	5
Acetate	+	1000	2300	+	1000	2500	+	500	500
Ethanol	+	250	1000	+	100	250	+	5	5

^aSymbols: +, good growth; ±, weak growth; n.r., not reduced.^bColumn A: growth without tellurite; column B: highest rate and completeness of K_2TeO_3 reduction (μg/ml); column C: minimal inhibitory concentration (μg/ml).^cR.O., rich organic medium.**TABLE BXII.α.94.** Major characteristics of *Erythromicrobium* representatives^a

Characteristic	<i>E. ramosum</i>	<i>"E. ezovicum"</i>	<i>"E. hydrolyticum"</i>
Cell shape	Bacilli, branched	Long bacilli	Bacilli, branched
Cell size (μm)	0.7–1.0 × 1.6–2.5	0.6–0.8 × 2.7–2.8	0.7–1.1 × 1.8–2.5
Color	Red-orange	Red-orange	Red-orange
Major carotenoid <i>in vivo</i> peaks, nm	466, 478	466, 478	466, 478
Utilization of:			
Glucose	+	+	+
Maltose	+	+	+
Acetate	+	+	+
Pyruvate	+	–	+
Butyrate	+	+	–
Malate	+	+	+
Citrate	+	+	+
Succinate	+	+	+
Lactate	+	+	+
Ethanol	+	+	+
Methanol	–	–	–
Hydrolysis of:			
Starch	–	–	+
Gelatin	–	–	+
Tween-80	–	–	+
Mol% DNA G + C content	64.2	62.5	65.2

^aSymbols: +, substrate is utilized; –, substrate is not utilized.

DIFFERENTIATION OF THE GENUS *ERYTHROMICROBIUM* FROM OTHER GENERA

Species of the genus *Erythromicrobium* are freshwater bacteria inhabiting cyanobacterial mats of warm springs. The cells of certain representatives exhibit morphological pleomorphism and the ability to divide by ternary fission because of real branching of the cells. *E. ramosum*, *"E. ezovicum"*, and *"E. hydrolyticum"* are very long rods that often produce characteristic thread-like cells (Figs. BXII.α.101 and BXII.α.103B). The specific carotenoid composition determines the major carotenoid absorption peaks of the intact cell absorption spectra at 466 and 478 nm, apparent as an intense red-orange color in liquid cultures (Yurkov et al., 1997). The photosynthetic apparatus of all *Erythromicrobium* species described so far is unique. In addition to the reaction center and light-harvesting complex I, it contains a new type of light-harvesting complex II, with absorption maxima at 798–800 nm and 832–833 nm. The presence of this light-harvesting complex II in *Erythromicrobium* distinguishes its *in vivo* absorption characteristics from the absorption spectra of other aerobic and anaerobic anoxygenic phototrophic genera.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *ERYTHROMICROBIUM*

Although they resemble each other physiologically and morphologically, *Erythromicrobium* species have some distinguishing features (Table BXII.α.94). All described species differing from each

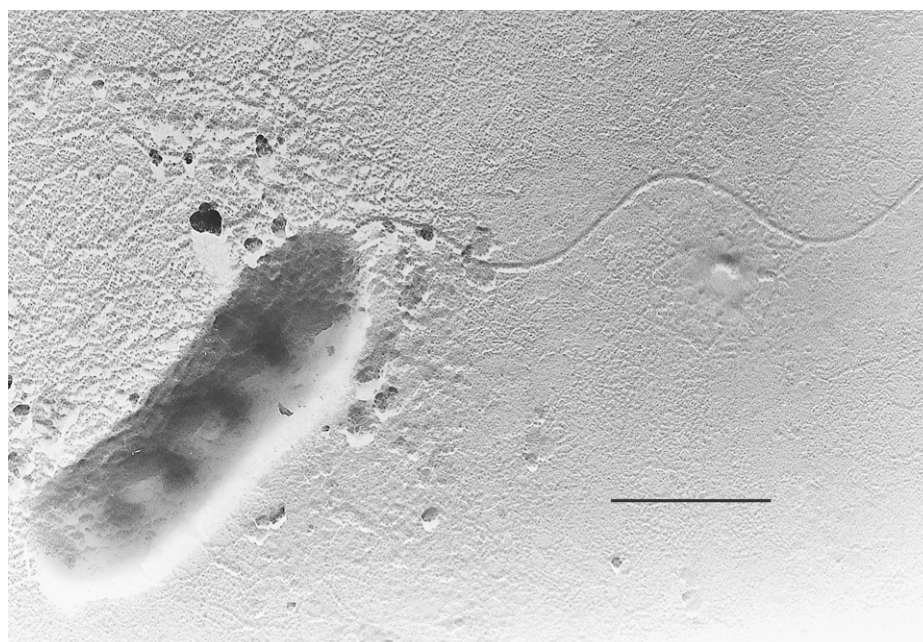
TAXONOMIC COMMENTS

The genus *Erythromicrobium* was introduced by Yurkov et al. (1992a) as a genus of freshwater obligately aerobic, facultatively photoheterotrophic bacteria that included five species: *E. ramosum*, *"E. ezovicum"*, *"E. hydrolyticum"*, *"E. sibiricum"*, and *"E. ursincola"*. The genus was validly published in 1994 with *E. ramosum* as the type strain (Yurkov et al., 1994c). In 1997, the genus *Erythromicrobium* was taxonomically reorganized, resulting in the exclusion of *"E. sibiricum"* and *"E. ursincola"* from the genus transfer to two new genera, *Sandaracinobacter* and *Erythromonas*, respectively (Yurkov et al., 1997).

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.
- Yurkov, V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

other by the mol% DNA G + C content and lower levels of DNA similarity, determined using DNA–DNA hybridization (Yurkov et al., 1991a). *"E. hydrolyticum"* demonstrates specifically high hy-



A



B

FIGURE BXII.α.103. "*Erythromicrobium ezovicum*". Electron micrographs. (Printed with permission from V. Yurkov.)
 A, Scanning micrograph showing polar flagellum. B, Long thread-like formation of cells. Bars = 1.0 μm.

drolytic activities utilizing starch, gelatin, and Tween-80 as sole carbon sources. This species can oxidize thiosulfate to tetrathionate in aerobic energy metabolism as an addition to using organic energy sources. Representatives of *Erythromicrobium* are resistant

to the toxic heavy metal oxide tellurite, reduce tellurite to tellurium, and accumulate metallic tellurium crystals inside the cells (Yurkov et al., 1996). However, the MIC (minimal inhibitory concentration) level of tellurite is species specific (Table BXII.α.93).

List of species of the genus Erythromicrobium

1. ***Erythromicrobium ramosum*** Yurkov, Stackebrandt, Holmes, Fuerst, Hugenholtz, Golecki, Gad'on, Gorlenko, Kompantseva and Drews 1994c, 432^{VP}

ra.mo'sum. L. adj. *ramosum* ramifying, referring to the morphology of the cells.

Gram-negative, red-orange rods that are 0.6–1.0 × 1.3–

2.5 μm . Cells may branch (Fig. BXII.α.101). Multiplication occurs by binary or ternary fission. Motile by means of polar flagella. Bacteriochlorophyll *a* and carotenoids are present. The cytoplasmic membrane contains a reaction center and two light-harvesting complexes, LHI with an absorption maximum at 870 nm and unusual "blue shifted" LHII with absorption maxima at 798 and 832 nm. The major carotenoids are the very polar compound erythroanthin sulfate and bacteriorubixanthin. Optimal growth occurs at temperatures between 25 and 30°C and at pH values between 7.0 and 8.5.

The optimal substrates for growth are glucose, sucrose, maltose, acetate, pyruvate, butyrate, malate, succinate, fumarate, propionate, glutamate, casein hydrolysate, and yeast extract. Growth also occurs on fructose, citrate, lactate, tartrate, and ethanol. No growth occurs on ribose, arabinose, formate, benzoate, mannitol, glycerol, and glycolate. Methanol is not utilized. Starch, gelatin, and Tween-80 are not hydrolyzed. Oxidase and catalase positive. The tricarboxylic acid cycle operates. The glyoxylate shunt has been observed in strain E4(2), but not in strain E5.

Susceptible to the following antibiotics: tetracycline, polymyxin B, erythromycin, nalidixic acid, amikacin, gentamicin, nystatin, bacitracin, kanamycin, neomycin, aureomycin, vancomycin, novobiocin, chloramphenicol, and fusidic acid. Resistant to penicillin, ampicillin, streptomycin, and nystatin. The storage compounds are polysaccharides, poly- β -hydroxybutyric acid, and polyphosphates.

Resistant to heavy metal oxide tellurite. Reduction of tellurite results in the accumulation of elemental tellurium inside cells. Tellurite resistance and reduction depend on supplemented organic carbon source (Fig. BXII.α.102; Table BXII.α.93).

Habitat: cyanobacterial mat from an alkaline spring (pH 9.5; 25°C).

The mol% G + C of the DNA is: 64.2 (T_m).

Type strain: E5, ATCC 700003, CIP 106927, DSM 8510, JCM 10282.

GenBank accession number (16S rRNA): AB013355 and X72909.

2. "**Erythromicrobium ezovicum**" Yurkov and Gorlenko 1992, 248.

e.zo'vi.cum. M.L. adj. *ezovicum* referring to the Russian river name Ezovca, site of isolation.

Gram-negative, red-orange colored rods, 0.6–0.8 \times 2.7–2.8 μm . Divide by symmetric or asymmetric constrictions. Nonbranching thread-like cells are characteristic. Motile by means of a polar flagellum (Fig. BXII.α.103). Cells produce bacteriochlorophyll *a* and carotenoids. *In vivo* absorption spectrum of bacteriochlorophyll *a* has maxima at 868 (light-harvesting complex I) and 798 nm, and a shoulder at 836 nm (light-harvesting complex II). Carotenoid acetone extracts have two peaks, at 466 and 478 nm.

Aerobic, chemoorganotrophic, facultative photoorganotrophic. No growth in the light under anaerobic conditions. Fermentation and denitrification activities not observed. The optimal temperature and pH are 25–30°C and 7.0–8.0, respectively.

Best growth in media containing glucose, sucrose, maltose, malate, succinate, casein hydrolyzate, and yeast extract. Good growth obtained on fructose, acetate, butyrate, cit-

rate, lactate, fumarate, propionate, tartrate, ethanol, and glutamate. Ribose, arabinose, pyruvate, formate, benzoate, methanol, mannitol, glycerol, and glycolate are not utilized. Gelatin, starch, and Tween-80 are not hydrolyzed.

A closed tricarboxylic acid cycle and the glyoxalate shunt operate. Oxidase and catalase positive.

Sensitive to the following antibiotics: tetracycline, polymyxin-B, amikacin, gentamicin, bacitracin, kanamycin, neomycin, aureomycin, vancomycin, chloramphenicol, and fusidic acid. Resistant to penicillin, ampicillin, streptomycin, erythromycin, nalidixic acid, lincomycin, mycostatin, and novobiocin.

High-level resistance to tellurite was established. However, unlike other species of *Erythromicrobium*, tellurite resistance of "*E. ezovicum*" is not always correlated with tellurite reduction to metallic tellurium (Table BXII.α.93). Other mechanisms, such as continuous tellurite efflux or tellurite complexing or methylation may play an important role in the resistance character. Tellurite is biotransformed to tellurium in glutamate-, succinate-, or malate-containing media (Yurkov et al., 1996).

Habitat: freshwater cyanobacterial mat that developed in alkaline spring (20–25°C; pH 9.5).

The mol% G + C of the DNA is: 62.5 (T_m).

Deposited strain: E-1.

3. "**Erythromicrobium hydrolyticum**" Yurkov and Gorlenko 1992, 249.

hyd.ro.ly'ti.cum. L. adj. *hydrolyticum* splitting water.

Gram-negative, red-orange rods 0.7–1.1 \times 1.8–2.5 μm or more. Cells may branch. Multiply by binary or ternary fission. Motility not observed. Cells contain bacteriochlorophyll *a* and carotenoids. Bacteriochlorophyll *a* in intact cells has major peak at 868 nm (indicating the presence of light-harvesting complex I), a minor peak at 798 nm, and a shoulder at 836 nm (indicating the presence of "blue shifted" light-harvesting complex II). Carotenoid acetone extracts have peaks at 466 and 478 nm.

Aerobic, chemoorganotrophic, facultative photoorganotrophic. Thiosulfate may be utilized as an additional source of energy, being oxidized to tetrathionate. Anaerobic growth in the light does not occur. Fermentation and denitrification activities are not detected. Favorable growth temperature, 25–30°C. Optimal pH, 7.0–8.0.

Best growth occurs in the presence of glucose, maltose, pyruvate, malate, succinate, fumarate, casein hydrolyzate, and yeast extract. Fructose, acetate, citrate, tartrate, and ethanol support a good growth. Lactate supports weak growth. Ribose, arabinose, butyrate, formate, propionate, benzoate, methanol, mannitol, glycerol, and glycolate cannot be used as organic carbon sources. Starch, gelatin, and Tween 80 are hydrolyzed.

Tellurite resistant. Transform tellurite to metallic tellurium (Table BXII.α.93).

The closed tricarboxylic acid cycle and the glyoxalate shunt operate. Oxidase and catalase positive. Storage compounds: polysaccharides and polyphosphates.

Habitat: cyanobacterial mat from an alkaline spring (20–25°C; pH 9.5).

The mol% G + C of the DNA is: 65.2 (T_m).

Deposited strain: E4(1).

Genus VI. *Erythromonas Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177^{VP}**

VLADIMIR V. YURKOV

E.ry.thro.mo' nas. Gr. adj. *erythrus* red; Gr. n. *monas* a unit, monad; M.L. fem. n. *Erythromonas* red monad.

Ovoid, Gram negative, motile by means of polarly flagellated cells. Do not form chains. Reproduce by **budding** or asymmetric division. The cells are **orange-brown due to carotenoid pigments**. **Contain bacteriochlorophyll *a***. **Aerobic, chemoorganotrophic, and facultatively photoheterotrophic. No photosynthetic growth occurs under anaerobic conditions.** NaCl is not required for growth.

The mol% G + C of the DNA is: 65.4.

Type species: ***Erythromonas ursincola*** Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177.

FURTHER DESCRIPTIVE INFORMATION

The monospecific genus *E. ursincola* is phylogenetically related to members of the *Alphaproteobacteria* that form a separate subline on the evolutionary tree of *Sphingomonas* with a 16S rDNA homology of 92.5% (Yurkov et al., 1997).

E. ursincola cells are ovoid ($0.8\text{--}1.0 \times 1.3\text{--}2.6 \mu\text{m}$) and reproduce by budding or asymmetric division. The carotenoid composition of the species, apparent as absorption peaks at 430, 458, and 485 nm of the intact cell absorption spectra, produces the characteristic orange-brown color of *E. ursincola*. Bacteriochlorophyll *a* (BChl *a*) is incorporated into the photosynthetic apparatus that is organized in the reaction center with tightly bound tetraheme cytochrome *c* and light-harvesting complex I absorbing at 867 nm. Under anaerobic conditions, no light-induced absorbance changes can be observed in the photosynthetic apparatus of *E. ursincola*. In contrast, when cells were grown aerobically, typical photochemical reactions were revealed confirming aerobic photosynthetic functionality of the reaction center (Yurkov et al., 1995, 1998a).

E. ursincola has a complex soluble (three cytochromes *c* of 6.5, 9.0, and 14.0 kDa) and membrane-bound (four cytochromes *c* of 14.3, 21.0, 24.0, and 40.0 kDa) cytochrome population (Yurkov et al., 1997). Interestingly, *E. ursincola* contains an unusually small soluble cytochrome *c* of 6.5 kDa. Such a small cytochrome *c* is rare in bacteria and is present in *Hydrogenobacter thermophilus* (6.0 kDa), *Methylomonas* strain A4 (4.0 kDa), and an aerobic phototrophic species, *Roseococcus thiosulfatophilus* (4.0 and 6.5 kDa). The quinone composition of *E. ursincola* is represented by the only quinone Q-10 (Gogotov and Gorlenko, 1995).

Similarly to other aerobic phototrophic bacteria, *E. ursincola* is highly resistant to tellurite and reduces this compound to tellurium apparent as intracellular crystals. However, the minimal inhibitory concentration of tellurite (2700 $\mu\text{g}/\text{ml}$) for *E. ursincola* in acetate medium is significantly higher than that found in other species (Yurkov et al., 1996).

ENRICHMENT AND ISOLATION PROCEDURES

Strain KR99 designated as *E. ursincola* was isolated from samples of a cyanobacterial mat that developed in freshwater thermal springs (pH 6.7–7.0 and temperature, 34–40°C). The Neskuchinskii spring is situated on Kunashir Island (Southern Kurily,

Russia). The techniques used to isolate and cultivate *E. ursincola* are similar to those described for *Erythromicrobium*.

MAINTENANCE PROCEDURES

Erythromonas can be preserved by standard procedures in liquid nitrogen, by freezing at -70 to -80°C or by lyophilization.

DIFFERENTIATION OF THE GENUS *ERYTHROMONAS* FROM OTHER GENERA

The genus *Erythromonas* is separated from other genera of the aerobic anoxygenic phototrophic bacteria by its distinguishing morphological, physiological, as well as genetic properties. Its comparatively large cell size, ovoid form, budding, and characteristic absorption spectrum of the intact cells are distinctive phenotypic properties.

The major morphological and physiological differences between *Erythromonas* and its most closely related genera of freshwater aerobic phototrophic genera are shown in Table BXII.α.95.

TAXONOMIC COMMENTS

Newly isolated in 1991, strain KR99 was initially assigned to the genus *Erythromicrobium* under the species name "*E. ursincola*" (Yurkov et al., 1991a). However, detailed phylogenetic investigation of 16S rDNA sequences and precise chemotaxonomic research performed on this bacterium later clearly revealed that a new genus named *Erythromonas* with *E. ursincola* as a type species should be created (Yurkov et al., 1997).

Phylogenetic comparisons of 16S rDNA sequences determined that *E. ursincola* clusters with *Blastomonas natatoria* (99.8% sequence identity) (Yurkov et al., 1997). However, significant phenotypic and physiological differences exist between *E. ursincola* and *B. natatoria* that preclude their assignment to the same genus. *B. natatoria* is a nonphotosynthetic species containing carotenoid pigments and lacking bacteriochlorophyll, whereas *E. ursincola* is physiologically related to obligately aerobic anoxygenic phototrophic bacteria producing carotenoids and BChl *a*. *E. ursincola* contains BChl *a* incorporated into a photochemically active reaction center and light-harvesting complexes, and contains specific electron transfer carriers of a cyclic photosynthetic pathway (such as cytochrome *c* bound to the reaction center, soluble cytochrome *c*₂, the reaction center Q_A primary electron acceptor, and the "special pair" P of the reaction center). High 16S rDNA sequence similarity between these two species indicates a close phylogenetic relationship and that they share a common ancestor; conceivably one is an evolutionary progenitor of the other. However, due to the occurrence of significant physiological differences (photosynthesis is a restricted mode of energy generation), they are not designated as members of the same genus for taxonomic purposes.

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.
- Yurkov, V.V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

*Editorial Note: The type and only species of the genus *Erythromonas* has been transferred to the genus *Sphingomonas*.

TABLE BXII.α.95. Determinative characteristics of taxonomically closely related freshwater aerobic anoxygenic phototrophic genera

Characteristics	<i>Erythromonas</i>	<i>Sandaracinobacter</i>	<i>Erythromicrobium</i>	<i>Porphyrobacter</i>
Cell shape	Ovoid	Thin, long rods	Rods, branched	Pleomorphic
Cell size, μm	0.8–1.0 \times 1.3–2.6	0.3–0.5 \times 1.5–2.5	0.7–1.0 \times 1.6–2.5	0.4–0.8 \times 1.1–2.0
Color	Orange-brown	Yellow-orange	Red-orange	Orange-red
Carotenoid <i>in vivo</i> peaks, nm	430, 458, 485	424, 450, 474	466, 478	464, 491
BChl <i>a in vivo</i> peaks, nm	800, 867	800, 867	798, 832, 868	799, 869
Mol% DNA G + C content	65.4	68.5	64.2	65–66

List of species of the genus Erythromonas

1. ***Erythromonas ursincola*** Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177^{VP}
ur.sin'co.la. M.L. adj. *ursincola* neighbor or compatriot of bears.

Cells are Gram-negative and ovoid 0.8–1.0 \times 1.3–2.6 μm . Long chains are not formed. Reproduce by budding or asymmetric division. Cells motile by means of a unique polar flagellum. Cells contain BChl *a* and carotenoid pigments. Carotenoids have three main absorption peaks at 430, 458, 485 nm *in vivo*, and in combination with BChl *a*, determine the culture color: orange in young culture and dark orange-brown in older liquid or young agar cultures. BChl *a* is present in membrane-bound, protein-pigment complexes, consisting of the reaction center with absorption peaks at 751, 801, and 853 nm and core light-harvesting complex I absorbing at 867 nm. Reaction center contains tightly bound tetraheme cytochrome *c* with a molecular weight of 40.0 kDa. Total cytochrome *c* composition of cells growing in the dark is very abundant and represented by soluble cytochrome *c* of 6.5, 9.0, and 14.0 kDa, and membrane-bound cytochrome *c* of 14.3, 21.0, and 24.0 kDa. In cells growing in the dark, only quinone Q-10 was determined.

Aerobic, chemoorganotrophic, facultatively photoorganoheterotrophic. Best growth is on media containing glucose, fructose, sucrose, maltose, acetate, glutamate, propionate, casein hydrolyzate, or yeast extract. Good growth on pyruvate, butyrate, malate, or succinate. Poor growth on media supplemented with arabinose, citrate, lactate, glycerol or mannitol. No growth detected in media containing

ribose, formate, benzoate, tartrate, methanol, ethanol, or glycolate. The tricarboxylic acid cycle and glyoxylate shunt operate during growth on acetate containing medium. The key enzyme of the Calvin cycle, ribulose diphosphate carboxylase, is not found. No anaerobic growth in the light, fermentation, or denitrification is found.

Optimal growth temperature is 25–30°C. Freshwater organism, does not require NaCl for growth. Optimal pH is 7.0–8.0. Yeast extract and vitamin B₁₂ satisfy the requirement for growth factors. Oxidase and catalase positive. Tween 80 is hydrolyzed. Lipase activity of 64.9 U/g of biomass. Starch and gelatin are not hydrolyzed. Sensitive to tetracycline, polymyxin B, amikacin, gentamicin, neomycin, aureomycin, vancomycin, novobiocin, chloramphenicol, and fusidic acid. Resistant to penicillin, ampicillin, streptomycin, erythromycin, nalidixic acid, lincomycin, mycostatin, bacitracin, and kanamycin. Very resistant to tellurite. Can grow in the presence of tellurite up to 2700 $\mu\text{g}/\text{ml}$ in acetate-containing minimal medium. Resistance to tellurite depends on organic carbon source in the medium. Tellurite can be reduced and transformed into metallic tellurium, which is accumulated as metal crystals in the cell cytoplasm.

Storage compounds: polyphosphates.

Habitat: freshwater cyanobacterial mat developing in the thermal springs at pH 6.7–7.0 and temperature 34–40°C.

The mol% G + C of the DNA is: 65.4 (*T_m*).

Type strain: KR-99, EY 4250, CIP 106843, DSM 9006, JCM 10397.

GenBank accession number (16S rRNA): Y10677, AB024289.

Genus VII. *Porphyrobacter* Fuerst, Hawkins, Holms, Sly, Moore and Stackebrandt 1993,
 132^{VP}

AKIRA HIRAISHI AND JOHANNES F. IMHOFF

Por.phy.ro.bac' ter. Gr. adj. *porphyreos* purple; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bacterion* a rod; M.L. masc. n. *Porphyrobacter* porphyrin-producing rod.

Cells are straight, pleomorphic, ovoid-to-short rods, or cocci, 0.5–1.0 \times 0.8–2.0 μm . Spores and capsules are not formed. Multiply by budding or binary fission. Gram negative. Members of the *Alphaproteobacteria*. Motile or nonmotile. Do not form any type of internal membranes. Synthesize bacteriochlorophyll *a* esterified with phytol and carotenoids as photosynthetic pigments only under aerobic conditions in the dark.

Strictly aerobic, chemoorganoheterotrophic bacteria containing bacteriochlorophyll. Do not grow phototrophically under anoxic conditions in the light. Simple organic compounds, peptone, and yeast extract used as electron donors and carbon sources. Produce acid but not gas from glucose. Catalase positive

and oxidase negative. Some strains may require vitamins for growth.

Mesophilic to moderately thermophilic, neutrophilic freshwater bacteria.

Straight-chain octadecenoic acid (C_{18:1}) is the major cellular fatty acid. 2-Hydroxy fatty acids and sphingoglycolipids are present. 3-Hydroxy fatty acids are absent. Ubiquinone-10 is the major quinone.

The mol% G + C of the DNA is: 65.0–66.4.

Type species: ***Porphyrobacter neustonensis*** Fuerst, Hawkins, Holms, Sly, Moore and Stackebrandt 1993, 132.

FURTHER DESCRIPTIVE INFORMATION

Based on 16S rDNA sequence analysis, *Porphyrobacter* species belong to the *Alphaproteobacteria*, with *Erythrobacter* and *Erythromicrobium* species as phylogenetic neighbors. The nearest phylogenetic neighbor is *Erythromicrobium ramosum*, with 98% 16S rDNA sequence identity to *Porphyrobacter neustonensis*.

Cell morphology of *Porphyrobacter* varies depending upon the species. The type species, *P. neustonensis*, contains rod-shaped and pleomorphic cells and in some cases exhibits coccoid forms (Fig. BXII.α.104). The cells reproduce by polar growth and budding, and this mode of cell division is similar to a division type found in *Planctomycetales*, in which the daughter cell appears as a small spherical bud. Cells produce multifibrillar stalk-like fascicle structures and crateriform structures on the cell surface. Cells of *P. neustonensis* are motile by means of subpolar or peritrichous flagella (Fig. BXII.α.105). Cells of *P. tepidarius* are ovoid-to-short

rods, nonmotile, and multiply by binary fission (Fig. BXII.α.106). Thin-section electron microscopy shows that *Porphyrobacter* species do not contain internal membranes (Fig. BXII.α.107). Thin-sections of *P. neustonensis* indicated the presence of an electron-dense cell wall layer in the mother cell but the absence of such a layer in the bud (Fig. BXII.α.108). Analyses of crude cell wall material of *P. neustonensis* showed that muramic acid and diaminopimelic acid were present in the cell wall (Fuerst et al., 1993).

A group of highly polar carotenoids, including carotenoid sulfates and bacteriorubixanthin, are major components (Hanada et al., 1997). Nostaxanthin is also present in *P. tepidarius*. Unlike *Erythromicrobium* and *Erythrobacter* species, *Porphyrobacter* species lack zeaxanthin. Absorption spectra of living cells or membrane preparations have a low absorption maximum at

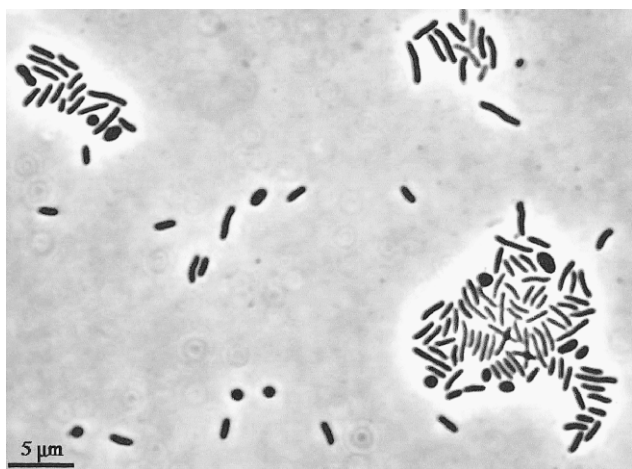


FIGURE BXII.α.104. Phase-contrast photomicrograph showing general cell morphology of *Porphyrobacter neustonensis*. Rod-shaped and coccoid cells coexist. Bar = 5 μm.



FIGURE BXII.α.106. Electron photomicrograph of negatively stained cells of *Porphyrobacter tepidarius*. Scale: 2.4 cm = 5 μm. (Reproduced with permission from S. Hanada et al., *International Journal of Systematic Bacteriology*, 47: 408–413, 1997 ©International Union of Microbiological Societies.)

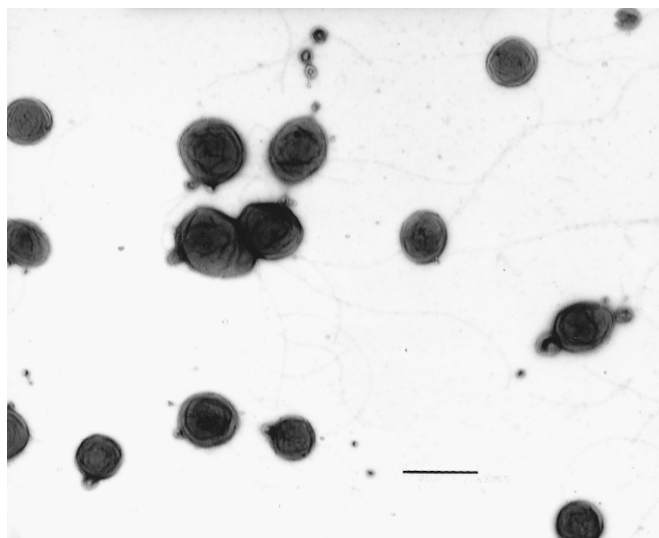


FIGURE BXII.α.105. Electron photomicrograph of negatively stained cells of *Porphyrobacter neustonensis*. Bar = 1 μm. (Reproduced with permission from J.A. Fuerst et al., *International Journal of Systematic Bacteriology*, 43: 125–134, 1993 ©International Union of Microbiological Societies.)



FIGURE BXII.α.107. Thin-section electron photomicrograph showing the ultrastructure of *Porphyrobacter tepidarius*. Scale 2.3 cm = 0.5 μm. (Reproduced with permission from S. Hanada et al., *International Journal of Systematic Bacteriology*, 47: 408–413, 1997 ©International Union of Microbiological Societies.)

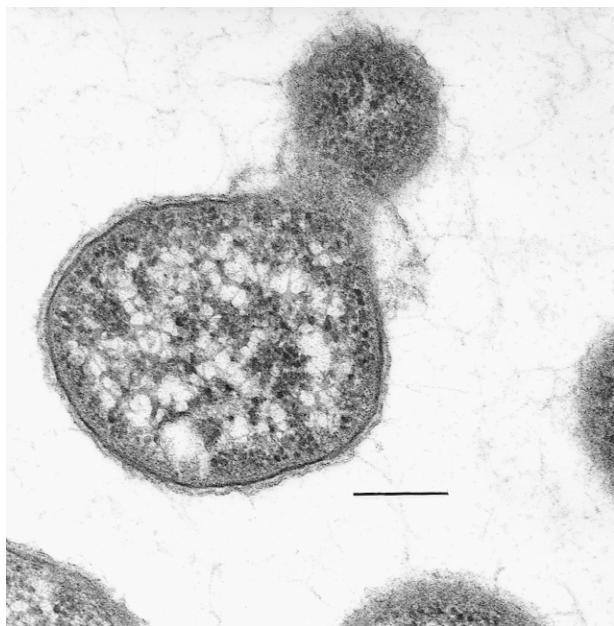


FIGURE BXII.α.108. Thin-section electron photomicrograph showing the ultrastructure of *Porphyrobacter neustonensis*. Bar = 0.2 μm. (Reproduced with permission from J.A. Fuerst et al., International Journal of Systematic Bacteriology, 43: 125–134, 1993 ©International Union of Microbiological Societies.)

around 800 nm and a high absorption maximum at 868–871 nm. These spectral patterns in the near-infrared region indicate that the cells contain the core light-harvesting (LH) complex (B870), together with the photosynthetic reaction center, and lack the peripheral light-harvesting complex LHII, as is the case in other aerobic bacteriochlorophyll-containing bacteria of the *Alphaproteobacteria*.

Like many other aerobic photosynthetic bacteria, *Porphyrobacter* produces BChl *a* only under aerobic growth conditions in darkness. The amount of BChl *a* produced by *P. neustonensis* ACM 2844 is maximal in gently stirred dark broth cultures (J.A. Fuerst, personal communication). Standing broth cultures produce 0.08 ± 0.05 nmol/mg dry wt of BChl *a*, whereas stirred cultures produce 1.04 ± 0.09 nmol/mg dry wt and shaken cultures 0.65 ± 0.10 nmol/mg dry wt. The maximum BChl *a* content of *P. neustonensis* is somewhat lower than, or comparable to, the BChl *a* content reported for the phylogenetic neighbor *Erythrobacter longus* (Harashima et al., 1982). Due to the presence of carotenoids, liquid cultures and colonies on agar media are orange to red. The intensity of pigmentation depends upon the growth medium. For *P. neustonensis*, casitone–yeast extract agar is best for pigment production (Fuerst et al., 1993).

Porphyrobacter species are aerobic chemoheterotrophic bacteria that grow well under aerobic dark conditions but not anaerobically in the light. They do not grow by anaerobic respiration with nitrate, dimethyl sulfoxide, or trimethylamine *N*-oxide as the terminal electron acceptor. Most strains exhibit a doubling time of 2–4 h under optimal growth conditions. Simple organic compounds such as glucose are used as electron donors and carbon sources for growth. Peptone and yeast extract are also good substrates. Hydrolytic activities against starch, chitin, gelatin, and DNA are absent, whereas Tween 80 hydrolysis is positive in all strains.

The lipid composition of *Porphyrobacter* has not yet been studied in detail, but some information on fatty acid profiles is available. The major cellular fatty acid is straight-chain octadecenoic acid (C_{18:1}). Some strains of *P. neustonensis* contain either C_{17:1} or C_{18:2} as a second major component. Characteristic for *Porphyrobacter* is the production of 2-OH fatty acid components and glycosphingolipids (A. Hiraishi, unpublished data). These properties are found in other genera of the *Alphaproteobacteria*, such as *Erythrobacter* and the obligately chemotrophic relative *Sphingomonas*. All *Porphyrobacter* species contain ubiquinone-10 as their sole respiratory quinones.

The natural habitats of *Porphyrobacter* are freshwater environments. *P. neustonensis* strains have been isolated from the air-water interface of freshwater bodies (Fuerst et al., 1993). *P. tepidarius* was isolated from cyanobacterial mats developing in the Usami hot spring (Shizuoka Prefecture, Japan), which contains brackish water (Hanada et al., 1997).

ENRICHMENT AND ISOLATION PROCEDURES

Growth media and cultural conditions commonly used for the isolation of freshwater aerobic bacteria can be used for the isolation of *P. neustonensis*. The surface water of freshwater environments is a possible source of this organism. As an isolation medium, casitone–yeast extract agar medium can be used (Fuerst et al., 1993).

The medium used for isolation of *P. tepidarius* is PE agar medium (Hanada et al., 1995, 1997), which includes acetate, succinate, glutamate, yeast extract, and Casamino acids as carbon sources. The medium is inoculated with water samples from thermal environments at 40–50°C and incubated at pH 5–8 and 45°C in darkness. After several days of incubation, orange colonies may appear on the agar medium.

MAINTENANCE PROCEDURES

Cultures are well preserved in liquid nitrogen or by lyophilization. Preservation in an electric freezer at –80°C is also possible.

DIFFERENTIATION OF THE GENUS *PORPHYROBACTER* FROM OTHER GENERA

Differential characteristics of the genus *Porphyrobacter* and the phylogenetically and phenotypically related genera *Erythromicrobium* and *Erythrobacter* are shown in Table BXII.α.96.

TAXONOMIC COMMENTS

Porphyrobacter neustonensis is the first organism described as a budding species of aerobic bacteriochlorophyll-containing bacteria (Fuerst et al., 1993). Phylogenetically and physiologically, *P. neustonensis* is closely related to *Erythromicrobium* and *Erythrobacter*, but differences in morphological properties and in natural habitats led to the proposal of a new genus for this species. Based on phylogenetic evidence, *P. tepidarius* and *P. neustonensis* are closely related species, although cell morphology and temperature relations distinguish the two (Hanada et al., 1997).

Intensive studies on the isolation and phylogenetic analysis of aerobic BChl-containing bacteria in freshwater and marine environments have yielded new budding strains in both the genera *Porphyrobacter* and *Erythrobacter* (A. Hiraishi, unpublished data; M. Suzuki, personal communication). In addition, the marine bacterium “*Agrobacterium sanguineum*” (Ahrens and Rheinheimer, 1967) has been shown to belong to the genus *Porphyrobacter* phylogenetically (A. Hiraishi, unpublished). In view of these observations and the close phylogenetic relationship of *Porphyrobacter*

TABLE BXII.α.96. Differential characteristics of the genus *Porphyrobacter* and related aerobic bacteria containing bacteriochlorophyll^a

Characteristic	<i>Porphyrobacter</i>	<i>Erythromicrobium</i>	<i>Erythrobacter</i>
Pleomorphic rods/cocci	D	—	—
Cell width (μm)	0.5–1.2	0.6–1.0	0.3–0.4
Motility by flagella	D	+	+
Carotenoid:			
Carotenoid sulfate	+	—	—
Erythroxanthin sulfate	—	+	+
Zeaxanthin	—	+	+
NaCl required for growth	—	—	+
Tween 80 hydrolysis	+	—	+
Habitat	Freshwater	Freshwater	Marine
Mol% G + C of DNA	65–67	64	57–67

^aSymbols: +, 90% or more of strains positive; —, 90% or more of strains negative; D, different reactions in different species.

species to *Erythromicrobium* (>98% similarity in 16S rDNA sequence) and *Erythrobacter* (>95% similarity), a taxonomic rearrangement of these genera may result from future studies.

ACKNOWLEDGMENTS

The authors are indebted to J.A. Fuerst and S. Hanada for their contribution of figures, unpublished information, and helpful discussion.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *PORPHYROBACTER*

General and differential characteristics of the two species *P. neustonensis* and *P. tepidarius* are included in Table BXII.α.97. The average level of genomic DNA–DNA hybridization between *P.*

neustonensis and *P. tepidarius* is 30%. The two species also show 99% 16S rDNA sequence similarity.

TABLE BXII.α.97. General and differential characteristics of the species of the genus *Porphyrobacter*^{a,b}

Characteristic	<i>P. neustonensis</i>	<i>P. tepidarius</i>
Cell shape	pleomorphic rods or cocci	ovoid-to-short rods
Cell division	budding	binary fission
Motility by flagella	+	—
Color of colonies	Orange, red	Orange
Optimal growth temperature (°C)	28–30	40–48
Growth with 3% NaCl	—	—
Vitamin required for growth	—	Biotin
Catalase activity	+	+
Oxidase activity	—	—
Nitrate reduction to nitrite	—	nd
Antibiotic susceptibility:		
Chloramphenicol (100 μg/ml)	+	+
Penicillin (20 U)	+	+
Streptomycin (50 μg/ml)	—	nd
Major quinone	Q-10	Q-10
Mol% G + C of DNA	65.7–66.4	65
Hydrolysis of:		
Starch	—	+
Chitin	—	nd
Esculin	d	nd
Casein	d	—
Gelatin	—	—
Tween 80	+	+
DNA	—	nd
Electron donor/carbon source:		
D-Xylose	+	nd
D-Glucose	+	+
D-Galactose	+	nd
D-Mannose	+	nd
D-Fructose	d	+

(continued)

TABLE BXII.α.97. (cont.)

Characteristic	<i>P. neustonensis</i>	<i>P. tepidarius</i>
Maltose	+	nd
Cellobiose	d	nd
Lactose	—	—
Sucrose	+	nd
Raffinose	d	nd
Trehalose	d	nd
Mannitol	d	nd
Methanol	—	—
Ethanol	—	—
Formate	—	—
Acetate	—	+
Butyrate	—	+
Pyruvate	+	—
Lactate	—	—
Citrate	—	—
Succinate	d	—
Fumarate	d	—
Malate	—	—
Benzoate	—	—
Alanine	d	nd
Asparagine	d	nd
Glutamate	—	+
Histidine	d	nd
Isoleucine	d	nd
Ornithine	d	nd
Phenylalanine	—	nd
Proline	+	nd
Yeast extract	+	+

^aSymbols: +, 90% or more of strains positive; —, 90% or more of strains negative; d, 11–89% of strains positive; nd, information unavailable; Q-10, ubiquinone-10.

^bSubstrates tested but not utilized by *P. neustonensis*: L-arabinose, D-ribose, L-rhamnose, inulin, adonitol, dulcitol, erythritol, sorbitol, caproate, caprylate, glycolate, tartrate, phthalate, leucine, lysine, methionine, phenylalanine, tyrosine, and valine.

List of species of the genus *Porphyrobacter*

1. ***Porphyrobacter neustonensis*** Fuerst, Hawkins, Holms, Sly, Moore and Stackebrandt 1993, 132^{VP}
neu.sto.nen'sis. Gr. n. *neustos* swimming, floating; M.L. masc. adj. *neustonensis* occurring at the air–water interface layer.

Cells are pleomorphic rods or cocci, $0.5\text{--}1.0 \times 0.8\text{--}2 \mu\text{m}$ and multiply by polar growth or budding. Produce multifibrillar, stalk-like, fascicle structures and crater-form structures on the cell surface. Motile by means of subpolar or peritrichous flagella. The absorption spectrum of living cells has maxima at 460, 494, 799–806, and 868–871 nm. Contain a group of highly polar carotenoids and bacteriorubixanthinal as the major components.

Obligately aerobic, chemoorganoheterotrophic bacterium. No phototrophic growth anaerobically in the light. Good growth occurs with xylose, glucose, galactose, mannose, maltose, sucrose, pyruvate, proline, and yeast extract. Methanol, ethanol, formate, acetate, lactate, and malate are not used. Tween 80, but not starch or gelatin, is hydrolyzed. Susceptible to penicillin G and chloramphenicol, but resistant to streptomycin. No vitamins required as growth factors.

Mesophilic freshwater bacterium. Optimal growth is at 28–30°C; growth range is 10–37°C. No growth in the presence of 1.3% NaCl.

Habitat: air–water interface of freshwater subtropical ponds.

The mol% G + C of the DNA is: 65.0–66.4 (HPLC).

Type strain: ACM 2844, DSM 9434.

GenBank accession number (16S rRNA): AB03327, L01785, M96745.

2. ***Porphyrobacter tepidarius*** Hanada, Kawase, Hiraishi, Takaichi, Matsuura, Shimada and Nagashima 1997, 413^{VP}
tep.i.dar'ius. L. n. *tepidarium* a warm bath; M.L. adj. *tepidarius* warm bathing.

Cells are ovoid to rod-shaped, $0.5\text{--}0.7 \times 0.8\text{--}1.4 \mu\text{m}$, nonmotile and multiply by binary fission. Internal membranes may be absent. Colonies and liquid cultures are orange due to the presence of carotenoids and bacteriochlorophyll *a*. The *in vivo* absorption spectra show maxima at 460, 494, 596, 800, and 870 nm. The main carotenoids are OH- β -carotene sulfate derivatives, nostoxanthin, and bacteriorubixanthinal.

Obligately aerobic, chemoorganoheterotrophic bacterium. No phototrophic growth anaerobically in the light. Good growth occurs with glucose, acetate, butyrate, glutamate, Casamino acids, and yeast extract as sole energy sources. Methanol, ethanol, pyruvate, malate, and succinate are not used. Starch and Tween 80, but not gelatin, are hydrolyzed. Susceptible to penicillin and chloramphenicol, but resistant to streptomycin. Biotin required as growth factor.

Moderately thermophilic freshwater bacterium, able to tolerate up to 1.3% NaCl. Optimal growth is at 40–48°C; growth range is 30–50°C. No growth in the presence of 2% NaCl.

Habitat: cyanobacterial mats in brackish water of hot springs (Shizuoka, Japan).

The mol% G + C of the DNA is: 65.0 (HPLC).

Type strain: OT3, DSM 10594.

GenBank accession number (16S rRNA): AB033328, D84429.

Genus VIII. *Sandaracinobacter* Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty* 1997, 1177^{VP}

VLADIMIR V. YURKOV

San.da.ra.ci'no.bac'ter. Gr. adj. *sandaracinos* orange-colored; Gr. n. *bacter* rod; M.L. masc. n. *Sandaracinobacter* orange-colored rod.

Cells are thin, long rods, forming chains. Motile by means of subpolar flagella. **Gram negative.** Divide by binary division. Cultures are **intensely yellow-orange because of carotenoid pigments.** **Contain bacteriochlorophyll *a*.**

Aerobic chemoorganotrophic and facultative photoheterotrophic metabolisms. No growth occurs anaerobically in light. Ribulose diphosphate carboxylase is not detected. No fermentation or denitrification activities observed. The habitat is fresh water. Not halophilic.

The mol% G + C of the DNA is: 68.5.

Type species: ***Sandaracinobacter sibiricus*** Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177.

FURTHER DESCRIPTIVE INFORMATION

Currently the genus *Sandaracinobacter* is represented by a single species, *S. sibiricus*. On the 16S rDNA tree, *Sandaracinobacter* forms a single subline of descent within the *Alphaproteobacteria* (Yurkov et al., 1997).

Thin, long ($0.3\text{--}0.5 \times 1.5\text{--}2.5 \mu\text{m}$), rod-shaped cells of *S. sibiricus* are intensely yellow-orange colored due to their high carotenoid content. The carotenoid composition, or at least the major pigments, of *S. sibiricus* (absorption at 424, 450, and 474 nm) are different from those detected for other aerobic phototrophic species and determine its unusual color. The cells of *S. sibiricus* often produce long chains (sometimes up to 10 cells) and reproduce by binary division. Budding and branching have not been observed. The cells are enclosed in a thin capsule. *S. sibiricus* is a nonhalophilic species, salt concentrations above 1% NaCl strongly inhibit bacterial growth.

S. sibiricus utilizes a variety of sugars such as glucose, fructose, sucrose, and maltose (Yurkov and Gorlenko, 1990). The catabolism of glucose by *S. sibiricus* occurs mainly via the Entner–Doudoroff pathway. The species possesses glucose-6-phosphate dehydrogenase and 2-keto-3-deoxygluconate-aldolase, two main enzymes of this pathway. Low activity of the key enzyme of the Embden–Meyerhof pathway, fructose-diphosphate-aldolase, was also detected in the cells growing in glucose-supplemented medium. It was concluded that this enzyme functions in biosynthesis. No 6-phosphogluconate dehydrogenase was detected in *S. sibi-*

*Editorial Note: Readers are advised that *Sandaracinobacter* and *Sandaracinobacter sibiricus* may be illegitimate names as the type material was not available from any public culture collection at the time this volume went to press.

cus, suggesting that the pentose monophosphate pathway does not function in this bacterium (Yurkov et al., 1992b).

The photosynthetic apparatus of *Sandaracinobacter* consists of a reaction center and one type of light-harvesting complex I (LHI), which absorbs maximally at 867 nm (Yurkov et al., 1997). The very fast cytochrome photooxidation observed after flash excitation in the cells of *S. sibiricus* indicates that the immediate electron donor to the reaction center in this species is a reaction center-bound cytochrome *c* (44.0 kDa) (Yurkov et al., 1995). Recently, reaction centers were purified from *S. sibiricus* membranes, confirming a typical overall organization of the reaction center (Yurkov et al., 1998a). The photochemical activity of the *S. sibiricus* photosynthetic apparatus was found to exist only under aerobic conditions (Yurkov et al., 1995, 1998a). Quinones Q-9 and Q-10 are the quinones of *S. sibiricus* (Gogotov and Gorlenko, 1995).

S. sibiricus demonstrated high-level resistance to toxic tellurite and accumulation of metallic tellurium crystals inside the cells. The level of tellurite resistance is strongly dependent on the organic carbon source used for growth. The highest resistance was found in yeast extract (sole carbon source)-containing medium and reached 1200 µg of tellurite per ml of medium (Yurkov et al., 1996).

S. sibiricus accumulates polyphosphates that provide a reserve of inorganic phosphate (Yurkov et al., 1991b). Osmium-stained granules of polyphosphates are found under nearly all experimental conditions studied—in the light and the dark, with high and low aeration. The highest amount of polyphosphate was accumulated in a growth medium supplemented with sucrose. Under such conditions, polyphosphate granules occupied about 30–40% of the total cell volume (Fig. BXII.α.109). Another in-

clusion body found in *S. sibiricus* consists of polyhydroxyalkanoate compound (a lipid-like compound that is formed from β-hydroxybutyric acid units) revealed as electron-transparent granules on electron micrographs (Yurkov et al., 1991b). Polyhydroxyalkanoate formation occurs when cells grow in media unbalanced for nitrogen (urea as a nitrogen source), as well as during incubation in a medium lacking fixed nitrogen. Replacement of ammonium with nitrate as the source of nitrogen also results in pronounced formation of polyhydroxyalkanoates with large granules occupying 40–50% of the total cell volume (Fig. BXII.α.110).

ENRICHMENT AND ISOLATION PROCEDURES

S. sibiricus was isolated from thin microbial mats that developed around the underwater hydrothermal vents of the Bol'shaya river bottom. The mats were formed by cyanobacteria *Oscillatoria subcapitata*, diatoms, and the purple bacteria *Thiocapsa roseopersicina* and *Rhodospseudomonas palustris*. The mats were situated at the boundary of the anaerobic and aerobic zones at pH 8.9–9.4, temperature 9–33°C, and hydrogen sulfide and oxygen concentrations of 0.6–4.5 mg/l and 10.4–4.1 mg/l, respectively. The water redox potential was high, $E_h = 230$ –330 mV. Thus, despite the presence of sulfide, the mat surface was washed by water with a high oxygen content (Yurkov and Gorlenko, 1990). Isolation procedures are identical to those described for the genus *Erythromicrobium*.

MAINTENANCE PROCEDURES

S. sibiricus can be readily maintained under short- and long-term storage following procedures described for the genus *Erythromicrobium*.

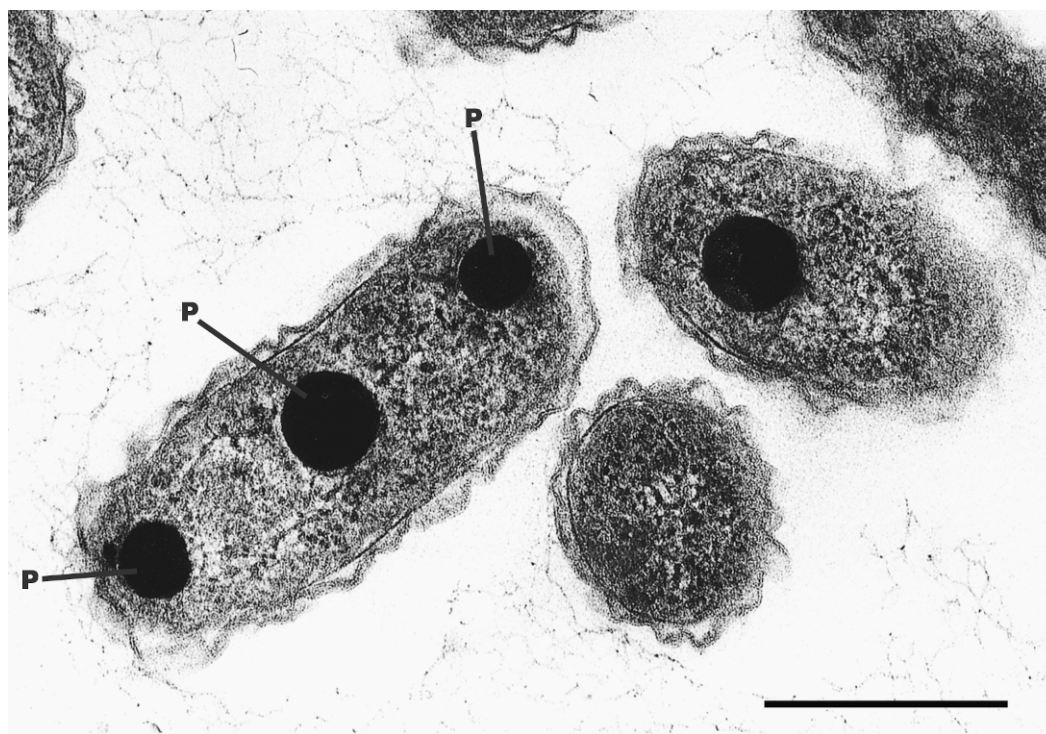


FIGURE BXII.α.109. Intracytoplasmic components of *S. sibiricus* revealed by electron microscopy of ultrathin sections. Electron dense granules of polyphosphates (*P* indicated by arrows). Bar = 0.5 µm. (Printed with permission from V. Yurkov).

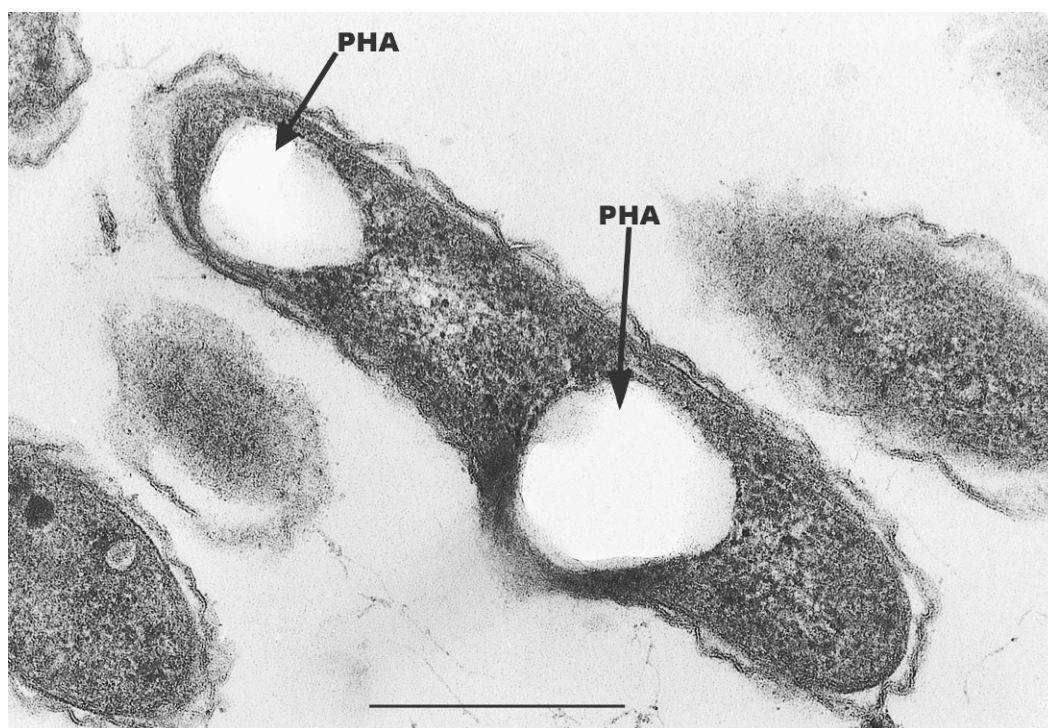


FIGURE BXII.α.110. *S. sibiricus*. Big electron clear granules of polyhydroxyalkanoate (PHA; indicated by arrows) often occupied up to 40–50% of the total cell volume. Note cell shape distortion by some granules. Bar = 0.5 μm. (Printed with permission from V. Yurkov.)

DIFFERENTIATION OF THE GENUS *SANDARACINOBACTER* FROM OTHER GENERA

Genomic difference of *Sandaracinobacter* from other genera of aerobic anoxygenic phototrophic bacteria was revealed by the DNA composition, DNA–DNA hybridization, and 16S rDNA sequence analyses. As determined by thermal denaturation, the mol% G + C content of *S. sibiricus* is 68.5. According to DNA–DNA hybridization, this species has a low level of DNA similarity with other species investigated. The highest level of DNA similarity (20–27%) was found with *Erythromonas ursincola* (formerly described as “*Erythromicrobium ursincola*”) (Yurkov et al., 1991a). Based on the 16S rDNA sequence data, *Sandaracinobacter* branches independently from its most closely related aerobic phototrophic genus *Erythromonas*, and nonphotosynthetic genera *Blastomonas*, “*Rhizomonas*”, and *Sphingomonas* (Yurkov et al., 1997).

The major morphological and physiological features of *Sandaracinobacter* that distinguish it from its closest relatives of freshwater aerobic phototrophic bacteria are shown in Table BXII.α.95 of the chapter describing genus *Erythromonas*.

TAXONOMIC COMMENTS

The first representative of a freshwater aerobic bacteriochlorophyll *a* (BChl *a*)-containing bacterium (strain RB16-17), isolated from thin microbial mats, was ascribed to the marine genus *Erythrobacter* and named “*Erythrobacter sibiricus*” (Yurkov and Gorlenko, 1990). This assignment was made based on bacteriochlorophyll *a* production and strict aerobiosis. However, since that time “*E. sibiricus*” was reclassified into the genus *Erythromi-*

crobium, a new genus for freshwater aerobic anoxygenic phototrophic bacteria (Yurkov and Gorlenko, 1992; Yurkov et al., 1991a, 1992a). Based on phenotypic similarities, the five species—“*E. sibiricus*”, “*E. ursincola*”, “*E. ezovicum*”, “*E. hydrolyticum*”, and *E. ramosum*—were included in the same genus, *Erythromicrobium* (Yurkov et al., 1991a). However, DNA–DNA hybridization data showed that DNA from the species “*E. sibiricus*” and “*E. ursincola*” had very low relatedness (11–27%) to the other three species of this genus. It was proposed that additional physiological, biochemical, and DNA sequence analyses might demonstrate clear differences between “*E. sibiricus*” and “*E. ursincola*” on the one hand, and the other orange freshwater bacteria, on the other hand (Yurkov et al., 1991a). In agreement with this proposal, an analysis of 5S rRNA sequences confirmed the genetic heterogeneity of the genus *Erythromicrobium* (Turova et al., 1995).

Recent results on the morphology, physiology, pigment composition, light-harvesting antenna, reaction center organization, and electron carriers of five *Erythromicrobium* representatives led to excluding “*E. sibiricus*” from the genus *Erythromicrobium* and, based on its unique phylogenetic position, describing this microorganism as the type species of a new genus, *Sandaracinobacter* (Yurkov et al., 1997).

FURTHER READING

- Shimada, K. 1995. Aerobic anoxygenic phototrophs. In Blankenship, Madigan and Bauer (Editors), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht. pp. 105–122.
- Yurkov, V.V. and J.T. Beatty. 1998. Aerobic anoxygenic phototrophic bacteria. *Microbiol. Mol. Biol. Rev.* 62: 695–724.

List of species of the genus Sandaracinobacter

1. **Sandaracinobacter sibiricus** Yurkov, Stackebrandt, Buss, Vermeglio, Gorlenko and Beatty 1997, 1177^{VP}
si.bi'ri.cus. L. adj. *sibiricus* isolated in Siberia.

Gram-negative, yellow-orange pigmented thin, long rods, $0.3\text{--}0.5 \times 1.5\text{--}2.5 \mu\text{m}$ or more. Motile by means of subpolar flagella (up to three). Cells contain BChl *a* and carotenoid pigments. Carotenoids have major absorption peaks at 424, 450, and 474 nm *in vivo*. The cytoplasmic membranes contain a reaction center (RC) and light-harvesting complex I (LHI) with absorption peaks at 750, 799, 857 nm for RC, and 867 nm for LHI. Tetraheme cytochrome *c* of 44.0 kDa is tightly bound to RC and is its immediate electron donor. Additionally contain soluble cytochrome *c* of 30.0 kDa (cytochrome of *bc₁* complex). Contain quinones Q-9 and Q-10. Menaquinone is not found.

Aerobic chemoorganotroph and facultative photoheterotroph. The best growth substrates are butyrate, sucrose, casein hydrolysate, and yeast extract. Good growth is observed on acetate and maltose; weak growth on media containing glucose, fructose, pyruvate, propionate, or glycerol. No utilization of ribose, sorbitol, benzoate, fumarate, formate, succinate, citrate, malate, methanol, or ethanol.

Optimal temperature for growth is 25–30°C. Freshwater bacteria; salinity above 1.0% of NaCl in media strongly inhibits growth. The pH optimum is 7.5–8.5. The bacteria exhibit oxidase activity and lack catalase activity. Hydrolyze Tween 60 and do not hydrolyze gelatin or starch.

Resistant to chloramphenicol, fusidic acid, streptomycin, amikacin, bacitracin, kanamycin, neomycin, and novobiocin. Sensitive to penicillin, ampicillin, tetracycline, polymyxin-B, erythromycin, nalidixic acid, lincomycin, mycostatin, aureomycin, and vancomycin.

Demonstrate a high level of resistance to tellurite. Tellurite resistance depends on medium composition, particularly on organic carbon source. The highest tellurite concentration tolerated is 1200 µg/ml in media containing acetate or yeast extract as a sole organic source. Tellurite can be reduced and transformed into metallic tellurium accumulated inside of cells.

Storage compounds: polyphosphates, polysaccharide, and polyhydroxyalkanoate.

Habitat: freshwater algobacterial mat near hydrothermal sulfide-containing vents along the river bottom.

The mol% G + C of the DNA is: 68.5 (*T_m*).

Type strain: RB16-17.

GenBank accession number (16S rRNA): Y10678.

Genus IX. *Zymomonas* Kluyver and van Niel 1936, 399^{AL}

GEORG A. SPRENGER AND JEAN SWINGS

Zy.mo'mo.nas or *Zy.mo.mo'nas*. Gr. n. *zyme* leaven, ferment; Gr. n. *monas* a unit, monad; M.L. fem. n. *Zymomonas* fermenting monad.

Rod-shaped cells with rounded ends, occasionally ellipsoidal, usually in pairs, $2\text{--}6 \times 1.0\text{--}1.4 \mu\text{m}$. Gram negative. **Usually non-motile; if motile, they possess one to four polar flagella.** Motility may be lost spontaneously. **Facultatively anaerobic; some strains are obligately anaerobic.** Optimum temperature 25–30°C. Colonies on the standard medium¹ are glistening, regularly edged, white to cream colored, 1–2 mm in diameter after 2 d at 30°C. Oxidase negative. Chemoorganotrophic, growing on and **fermenting 1 mol of glucose or fructose to almost 2 mol of ethanol, 2 mol of CO₂, and some organic acids such as lactic acid.** Some strains may also utilize sucrose, but other carbon sources are not used. Gluconate can be degraded but does not serve as sole carbon or energy source (Strohdeicher et al., 1988). Sorbitol and gluconolactone are formed when grown on sucrose or mixtures of glucose and fructose by a so far unique enzyme, glucose-fructose oxidoreductase. **Membranes contain pentacyclic triterpenoids of the hopane series** (Sahm et al., 1993). Gelatinase negative. Nitrates are not reduced and indole is not produced. ***Zymomonas* tolerates 5% ethanol and is acid tolerant, growing at pH 3.5–7.5.**

1. The standard medium (SM) has the following composition (g/l distilled water): D-glucose, 20; and yeast extract, 5. Another medium (RM) was introduced by Bringer et al. (1985) (g/l distilled water): D-glucose, 20; yeast extract, 10; KH₂PO₄, 1.0; (NH₄)₂SO₄, 1.0; and MgSO₄·7H₂O, 0.5. A defined medium (Fein et al., 1983) consists of (g/l distilled water): glucose, 20; KH₂PO₄, 3.5; (NH₄)₂SO₄, 2.0; MgSO₄·7H₂O, 1.0; FeSO₄·7H₂O, 0.01; and 2-[N-Morpholino]ethanesulfonate (MES), 19.52. The medium is adjusted to pH 5.5 and autoclaved. A sterile solution of biotin and Ca-pantothenate is added after autoclaving to a final concentration of 0.001 g/l each.

Good growth is obtained only when a mixture of amino acids is present in the medium, but no one amino acid is essential. All strains require biotin and pantothenate. ***Zymomonas* occurs as a spoiler in beers, ciders, and perries; as fermenting agents in Agave sap, palm sap, and sugarcane juice; and on honeybees and in ripening honey.**

The mol% G + C of the DNA is: 47.5–49.5.

Type species: Zymomonas mobilis (Lindner 1928a) Kluyver and van Niel 1936, 399 (*Thermobacterium mobile* Lindner 1928b, 253; *Zymomonas anaerobia* Kluyver 1957, 199.)

FURTHER DESCRIPTIVE INFORMATION

Morphology *Zymomonas* cells are mostly straight rods with rounded or ovoid ends, occurring singly or in pairs. *Zymomonas* cells form no spores, and contain no detectable intracellular lipids, glycogen, or poly-β-hydroxybutyrate. Some individual strains form rosette-like cell aggregations, cell chains, curved or U-shaped cells, or filamentous cells. Most strains are nonmotile.

Oxygen relationships Although *Zymomonas* has a fermentative type of metabolism, it is able to grow aerobically, and therefore should be qualified as a facultative anaerobe. Strains deficient in alcohol dehydrogenase are obligate aerobes (Wecker and Zall, 1987). An electron transport chain is present in the cytoplasmic membrane (Strohdeicher et al., 1990; Kalnenieks et al., 1993; Kim et al., 1995) but no oxidative phosphorylation occurs in the presence of glucose (Kalnenieks et al., 1993).

Cultural characteristics Deep colonies in solid standard me-

dium are lenticular, regular, entire edged, butyrous, white or cream colored, and 1–2 mm in diameter after 2–4 d at 30°C. Anaerobic surface colonies are spreading, entire edged, convex or umbonate, and 1–4 mm in diameter after 2–7 d at 30°C (Swings and De Ley, 1977). When incubated aerobically, colonies reach a maximum diameter of 1.5 mm or appear as microcolonies (Swings et al., 1977).

Most (90%) of the *Zymomonas* strains are able to grow between pH 3.85 and pH 7.55. At pH 3.5, 43% of the strains develop, indicating a high acid tolerance (Swings and De Ley, 1977). This feature is not at all surprising as the natural niche of the genus is in acid palm wines, ciders, and beers at pH 4 or below. *Zymomonas* cannot grow in liquid standard medium at pH 3.05.

Zymomonas grows best between 25°C and 30°C. At 38°C, 74% of the strains grow, but at 40°C, growth is rare (De Ley and Swings, 1976; Swings and De Ley, 1977). *Zymomonas* slowly develops at 15°C (Millis, 1951; Dadds et al., 1973) but not at 4°C. Growth at 36°C is the best phenotypic test to differentiate *Z. mobilis* subsp. *mobilis* (+) from *Z. mobilis* subsp. *pomacii* (–) (Swings et al., 1977). *Zymomonas* is killed by exposure to 60°C for 5 min.

Glucose metabolism *Zymomonas* grows easily in liquid media containing either D-glucose or D-fructose; a dense turbidity accompanied by abundant CO₂ formation develops after 1–2 d at 30°C. The final pH in the standard medium after 3 d at 30°C is 4.8–5.2. The acidification of the medium is more pronounced upon incubation at higher temperatures. Strain-specific flocculent or compact cell deposits are formed. Half of the strains grow in glucose concentrations up to 40% (Swings and De Ley, 1977).

Zymomonas is an unusual bacterium in that it ferments glucose anaerobically by the Entner–Doudoroff pathway (for references, see Conway, 1992), followed by a pyruvate decarboxylation and reduction of acetaldehyde, according to the following general fermentation balance:

$$1 \text{ glucose} \rightarrow (1.58\text{--}1.93) \text{ ethanol} + (1.7\text{--}1.9) \text{ CO}_2 + (0.02\text{--}0.2) \text{ lactate or other organic acid} + (0.011) \text{ cell material [CH}_2\text{O]}.$$

Small amounts of acetaldehyde, acetyl methyl carbinol, and glycerol are also formed. During the dissimilation of glucose under aerobic conditions, ethanol, acetaldehyde, and acetate are formed. In the presence of oxygen, NADH (from fermentation) is used by a membrane-bound NADH oxidase to reduce oxygen to water. Consequently, less NADH is available for the reduction of acetaldehyde to ethanol, and the accumulating acetaldehyde is growth inhibiting (Bringer et al., 1984; Pankova et al., 1985). Some acetate is formed from acetaldehyde through an NADP-dependent acetaldehyde dehydrogenase, with highest activity after aerobic growth (Barthel et al., 1989). Strains deficient in alcohol dehydrogenase are obligate aerobes (Wecker and Zall, 1987). A membrane-bound glucose dehydrogenase containing pyrroloquinoline-quinone as a prosthetic group (Strohdeicher et al., 1988), feeds the electrons derived from glucose into the respiratory chain, which contains ubiquinone-10 (Strohdeicher et al., 1990; Kalnietieks et al., 1993). A membrane-associated F₀F₁-ATPase has been purified (Reyes and Scopes, 1991), and ATP yields via oxidative phosphorylation have been shown with aerated cells using ethanol or acetaldehyde as substrates, but not with glucose (Kalnietieks et al., 1993). All enzymes from the Entner–Doudoroff pathway, including pyruvate decarboxylase and alcohol dehydrogenases, of *Z. mobilis* have been purified, the respective genes have been cloned, and the sequences thereof

have been established (Scopes et al., 1985; Pawluk et al., 1986; Aldrich et al., 1992; Conway, 1992; Sprenger et al., 1993).

Only a truncated tricarboxylic acid cycle is functional in *Zymomonas*. Malate and oxaloacetate are provided by action of a phosphoenolpyruvate carboxylase and a malic enzyme (Dawes et al., 1970; Bringer-Meyer and Sahm, 1989). From the pentose-phosphate pathway enzymes, transaldolase is missing (Feldmann et al., 1992; Zhang et al., 1995). Acetyl-CoA for anabolism is provided by an anaerobically active pyruvate dehydrogenase complex (Bringer-Meyer and Sahm, 1993; Neveling et al., 1998).

Only 2% of the glucose is incorporated in the cells, producing 48% of the cellular carbon (Belaïch and Senez, 1965); the rest of the carbon is derived from yeast extract components. The growth yield coefficients ($Y_{\text{glucose}} = 3.88\text{--}9.32$, as determined by several authors) indicate that the growth of these organisms is not very efficient.

Sucrose metabolism Sucrose is fermented and used for growth by many *Zymomonas* strains (for references, see Preziosi et al., 1990; Sprenger, 1996). This property is lost occasionally upon subculturing on D-glucose (Shimwell, 1950). Sucrose fermentation is inducible (Kluyver and Hoppenbrouwers, 1931; Dadds et al. 1973; Richards and Corbey, 1974) and can lead to capsule formation (Kirk et al., 1994): 35g/l of levan is formed from 150 g/l of sucrose (Yoshida et al., 1990). Other by-products of sucrose fermentation are fructo-oligosaccharides, sorbitol, and gluconate (Leigh et al., 1984; Viikari, 1984). Sorbitol and gluconolactone are formed by an intermolecular oxidation-reduction catalyzed by a periplasmic NADP-containing glucose-fructose oxidoreductase that so far has been detected only in *Zymomonas* (Zachariou and Scopes, 1986; Loos et al. 1991; G. A. Sprenger, unpublished results). Sorbitol is used as compatible solute and is accumulated intracellularly at high osmotic stress conditions (Loos et al., 1994). Sucrose is split extracellularly by action of levansucrase or invertase.

Glucose and fructose are taken up by facilitated diffusion through a transport protein that prefers glucose to fructose. *Zymomonas* appears to be the only known bacterium that relies solely on such a uniport type for sugar uptake (DiMarco and Romano, 1985; Parker et al. 1995; Weisser et al. 1995, 1996). Some strains form mannitol from fructose (Viikari, 1984). Dihydroxyacetone and glycerol are excreted after growth under suboptimal conditions with high fructose in the medium (Viikari, 1984) by the subsequent action of a dihydroxyacetone phosphate phosphatase and dihydroxyacetone reductase (Horbach et al., 1994).

Nitrogen sources The nitrogen source for growth can be supplied as peptone, yeast extract, nutrient broth, beer, palm juice, or apple juice or a mixture of 20 amino acids. (Groups of amino acids, individual amino acids, NH₄Cl, or (NH₄)₂SO₄ can also serve as nitrogen sources, but this has not been verified for every *Zymomonas* strain.) In synthetic media, the growth yield is lower and the generation time longer than in complex media, but the ethanol yield remains constant (Belaïch and Senez, 1965).

Growth factors *Zymomonas* strains require biotin and pantothenate as growth factors. No strain requires nicotinic acid. Only six strains need additional growth factors. The most exacting strain (VP3) requires additional vitamin B₂, lipoic acid, riboflavin, and folic acid (Van Pee et al., 1974).

Ethanol tolerance *Zymomonas* grows in the presence of 5% ethanol, and many strains grow at even higher concentrations

(Swings and De Ley, 1977; Bringer-Meyer and Sahm, 1988). The organism can produce high concentrations of ethanol, up to 13% (w/v) in batch cultures (Rogers et al., 1982), making it an attractive organism for industrial ethanol production. The main targets for detrimental action of ethanol are the cell membrane (Carey and Ingram, 1983) and fermentative activity; at an ethanol concentration of 10%, 3-phosphoglycerate is accumulated intracellularly concomitant with a decrease in fermentative activity (Strohhäcker et al., 1993). High ethanol levels or heat stress induces a characteristic protein pattern in the cells (Michel and Starka, 1986). A major protective function against ethanol can be ascribed to the hopanoids, pentacyclic triterpenoids, which occur in large amounts (up to 3% of membrane dry weight or 40–50% of total lipid content) in the membrane (Bringer et al., 1985; Flesch and Rohmer, 1989; Hermans et al., 1991; Sahm et al., 1993). Isoprenoids are synthesized exclusively via the pyruvate/glyceraldehyde-3-phosphate pathway (Rohmer et al., 1993). Other major membrane constituents are vaccenic acid (up to 70% of fatty acids; Tornabene et al., 1982; Carey and Ingram, 1983), sphingolipids as the precursor 3-oxosphinganine (Sutter, 1991) or free ceramide (*N*-palmitoyl-dihydroxyhexadecane; Tahara and Kawazu, 1990), and the newly discovered dialkylcyclohexadiene-carbinol (Koukkou et al., 1998). No evidence for ketodeoxyoctulosonic acid, heptoses, or hydroxy fatty acids has been found (Tornabene et al., 1982).

Other physiological features The following physiological tests are positive for the genus: catalase and superoxide dismutase (Bringer et al., 1984; Pankova et al., 1985; Shvinka et al., 1989); reduction of methylene blue, thionin, and 2,3,5-triphenyltetrazolium chloride; formation of traces of acetyl methyl carbinol and the production of a characteristic fruity odor. The following tests are negative: growth in 0.5% yeast extract, nutrient broth, or 1% peptone broth, in liquid standard medium + 2% NaCl; indole production, nitrate reduction, hydrolysis of gelatin, hydrolysis of Tween 60 and Tween 80, and oxidase.

Pathogenicity *Zymomonas* is not known to be pathogenic for humans, animals, or plants. Lindner (1929, 1931) recommended the use of *Zymomonas* in human nutrition as a kind of yogurt. Antagonistic effects of *Zymomonas* against bacteria and fungi *in vitro*, and the therapeutic use of *Zymomonas* in cases of chronic enteric and gynecological infections, have been reported (Swings and De Ley, 1977; Falcao de Moraes et al., 1993).

Ecology In sweet English ciders, *Zymomonas* is the causative agent of a secondary fermentation, known as "cider sickness". The first description of this phenomenon was given by Barker and Hillier (1912). Cider sickness is recognized by frothing and abundant gas formation, a typical change in the aroma and flavor, reduction of sweetness, and marked turbidity forming a heavy deposit afterward (Barker, 1948; Millis, 1951; Carr and Passmore, 1971). A cider disorder known as framboise, or "framboisement" in France, is also attributed to *Zymomonas*. Millis (1951) also isolated *Zymomonas* from "sick" perries. Lindner (1928b) discovered that *Zymomonas* is the fermentative agent that transforms the sugary *Agave* sap (aguamiel) to pulque in Mexico. *Zymomonas* is a serious beer contaminant, particularly in English cask beers, producing a heavy turbidity and an unpleasant odor due to acetaldehyde and H_2S . Dennis and Young (1982) isolated five strains of *Zymomonas* from an ale brewery using an isolation medium containing actidione and Schiff's reagent as a dye to detect acetaldehyde production. *Zymomonas* has not been reported in

lager beers. Palm wines are prepared in the Far East and in Africa from the sap of *Arenga*, *Raphia*, and *Elaeis* palms and are known to harbor *Zymomonas* as a fermentative agent. *Zymomonas* is also present in fermenting sugarcane juice in Brazil, and on bees and ripening honey in Spain.

Plasmids Indigenous plasmids in a size range of 1.5 kb to >40 kb of DNA have been described. Some antibiotic or heavy metal resistance features have been ascribed to the presence of these plasmids (for references, see Sprenger et al., 1993). Indigenous plasmids have been used to construct shuttle vectors for the expression of homologous and heterologous genes in *Z. mobilis* for metabolic engineering (Conway et al. 1987; Sprenger et al., 1993).

Potential for industrial applications and metabolic engineering *Zymomonas* may be important as an industrial ethanol producer and it offers advantages over traditional yeast fermentation: it has higher specific rates of glucose uptake and ethanol production; it gives higher ethanol yield and lower biomass; it grows anaerobically; and it has a high ethanol tolerance. Some authors have studied extensively the kinetics of ethanol fermentation in both batch and continuous cultures at high glucose concentrations (Lee et al., 1979; Rogers et al., 1982). Attempts to broaden the narrow substrate and product spectrum of *Z. mobilis* have yielded strains that can utilize carbon sources such as mannitol (Buchholz et al., 1988) after chemical mutagenesis. Introduction of heterologous genes from various microorganisms has led to strains that can utilize D-xylose (Feldmann et al., 1992; Zhang et al., 1995), D-mannose (Weisser et al., 1996), L-arabinose (Deanda et al., 1996), β -glucosides, or lactose (Sprenger, 1993). Similarly, strains that produce L-alanine (Uhlenbusch et al., 1991) or carotenoids (Misawa et al., 1991) have been described. Several reviews discuss the use of *Zymomonas* in the production of bioethanol from renewable carbon sources (Buchholz et al., 1987; Ingram et al., 1989; Johns et al., 1992; Sahm et al., 1992; Doelle et al., 1993; Sprenger, 1993, 1996; Sprenger et al., 1993).

ENRICHMENT AND ISOLATION PROCEDURES

The following medium, originally designed as a detection medium for *Zymomonas* in breweries (Dadds, 1971), can be recommended for enrichment and has the following composition (g/l distilled water): malt extract, 3; yeast extract, 3; D-glucose, 20; peptone, 5; and actidione, 0.02. The pH is adjusted to 4.0. Ethanol is added to a final concentration of 3% (v/v). The presence of *Zymomonas* is indicated by abundant gas production after 2–6 d incubation at 30°C.

MAINTENANCE PROCEDURES

Zymomonas cultures held in the standard medium at room temperature are transferred every 2–3 weeks. *Zymomonas* survives the ordinary lyophilization procedure for many years. Cell viability is rapidly lost in ionic dilution buffers as sodium citrate, sodium chloride, or sodium phosphate. A 10 mM Tris buffer (pH 8) containing 33 mM $MgCl_2$ should be used for serial dilutions at low temperatures (Buchholz and Eveleigh, 1989).

DIFFERENTIATION OF THE GENUS *ZYMONOMAS* FROM OTHER GENERA

Table BXII.α.98 indicates the most salient features that differentiate *Zymomonas* from other genera. *Zymomonas* can be differentiated from *Sphingomonas* by the absence of the fatty acid

C_{14:0} 2OH, which is typically present in all *Sphingomonas* species (Yabuuchi et al., 1990a; Takeuchi et al., 1993a; Balkwill et al., 1997), and also by the much lower mol% G + C of its DNA. *Zymomonas* is phenotypically and genotypically well defined and is easily recognized. Its most outstanding feature is the quantitative fermentation of glucose or fructose (and to a lesser extent of sucrose)—but no other sugars—to equimolar amounts of ethanol and CO₂. This feature makes *Zymomonas* a unique ethanol-producing bacterium.

Zymomonas is excluded from the *Enterobacteriaceae* based on polar flagellation, inability to reduce nitrates, growth at pH 4, and growth in the presence of 5% ethanol.

Genetically, phenotypically, and ecologically, *Zymomonas* is distantly related to the acetic acid bacteria: they both occur in acid, sugary, and alcoholized niches such as tropical plant juices and beer. They are ecologically complementary in that *Zymomonas* produces ethanol, which is further oxidized by the acetic acid and bacteria. The acetic acid bacteria are differentiated from *Zymomonas* by their strictly aerobic growth requirements and the mol% G + C values of their DNA.

TAXONOMIC COMMENTS

The genus *Zymomonas* belongs to the family *Sphingomonadaceae* based on phylogenetic analysis of 16S rRNA gene sequences (Balkwill et al., 1997). The genus *Zymomonas* contains only one species: *Zymomonas mobilis*. Strains ATCC 29192, NCIB 8777, and

10565 are almost identical and are united in *Zymomonas mobilis* subsp. *pomacii* (Swings et al., 1977). All the other strains belong in *Zymomonas mobilis* subsp. *mobilis*.

All *Zymomonas* strains have mol% G + C values for their DNA within the narrow range of 47.5–49.5. The tightness of the genus *Zymomonas* is also reflected in the high phenotypic similarity ($S_{\text{sim}} \geq 88\%$) between the strains (De Ley and Swings, 1976). DNA–DNA hybridizations show a nucleotide sequence similarity of >76%; only one strain, ATCC 29192, is aberrant with less than 32% DNA duplexing (Swings and De Ley, 1975). Because of the high phenotypic similarity, the authors refrained from proposing the subspecies *Zymomonas mobilis* subsp. *pomacii* as a separate species, although the DNA–DNA hybridization with reference strain 5.3 was very low (i.e., 32%), so low a value that it would have allowed the creation of two separate species. However, no additional strains and hybridization values were available to further support the separation. The homogeneity of the genus *Zymomonas* is further demonstrated by the computer-assisted comparison of electropherograms of the soluble cell proteins. The genome size has been determined to be in the range of $1.4\text{--}1.5 \times 10^9$ Da (Swings and De Ley, 1975; Kang and Kang, 1998).

Plasmid profiles from both *Z. mobilis* subspecies have been described and have been used to discriminate laboratory strains (Tonomura et al., 1982; Stokes et al., 1983; Skotnicki et al., 1984; Walia et al., 1984; Scordaki and Drainas, 1987; Yablonsky et al., 1988; Misawa and Nakamura, 1989; Degli-Innocenti et al. 1990).

List of species of the genus *Zymomonas*

1. ***Zymomonas mobilis*** (Lindner 1928a) Kluver and van Niel 1936, 399^{AL} (*Thermobacterium mobile* Lindner 1928b, 253; *Zymomonas anaerobia* Kluver 1957, 199.)
mo' bi. lis. L. adj. *mobilis* movable, motile.

The description of the species is as for the genus and as listed in Tables BXII.α.98 and BXII.α.99. The differentiation of its two subspecies is indicated in Table BXII.α.100.

The mol% G + C of the DNA is: 47.5–49.5 (T_m).

Type strain: ATCC 10988, DSMZ 424.

Additional Remarks: phenotypic centrotypic ATCC 29191.

- a. ***Zymomonas mobilis* subsp. *mobilis*** (Lindner 1928a) De Ley and Swings 1976, 156^{AL} (*Zymomonas mobilis* biovar anaerobia Richards and Corbey 1974, 243; *Zymomonas mobilis* biovar recifensis Gonçalves de Lima, De Araújo, Schumacher and Cavalcanti Da Silva 1970, 3; *Zymomonas anaerobia* biovar anaerobia (Shimwell 1937) Carr 1974, 353; *Saccharomonas anaerobia* biovar immobilis Shimwell 1950, 182; *Zymomonas anaerobia* biovar immobilis (Shimwell 1937) Carr 1974, 353.)
mo' bi. lis. L. adj. *mobilis* movable, motile.

TABLE BXII.α.98. Differential characteristics of the genus *Zymomonas* and other genera^a

Characteristics	<i>Zymomonas</i>	<i>Acetobacter</i>	<i>Aeromonas</i>	<i>Gluconobacter</i>	<i>Sphingomonas</i>	<i>Vibrio</i>
Gram variability occurs	—	+	—	+	—	—
Flagellar arrangement:						
Polar only	+	—	+	+	+	+
Peritrichous	—	+	—	—	—	—
Oxygen tolerance:						
Growth under both aerobic and anaerobic conditions	+	—	+	—	—	+
Growth under aerobic conditions only	—	+	—	+	+	—
Oxidase	—	—	+	—	+	+ ^b
Carbohydrate metabolism:						
Fermentative and respiratory	+	—	+	—	—	+
Respiratory only	—	+	—	+	+	—
Gas from D-glucose	+	—	D	—	—	—
1 mol of glucose fermented to 2 mol of ethanol and 2 mol of CO ₂	+	—	—	—	—	—
Nitrate reduction	—	—	+	—	—	+
Growth factors required	+ ^c	+ ^d	—	—	—	—
Growth at pH 4.0	+	+	—	+	—	—
Inhibited by novobiocin	+	D	—	+	—	d
Mol% G + C of DNA	47.5–49.5	51–65	57–62	56–64	60–65.4	38–51

^aSymbols: +, typically positive; —, typically negative; D, differs among species.

^bSome species exhibit a negative or weak oxidase reaction.

^cPantothenate and biotin.

^dPantothenate and/or niacin.

TABLE BXII.α.99. Other characteristics of *Zymomonas mobilis*^a

Characteristics	Reaction or result
Occurrence of "fruity" odor when cultured in standard medium	+
Growth in 0.5% yeast extract broth, in 0.5% peptone broth, or in beer	—
Growth in beer containing 2% glucose or on malt agar	+
<i>Salt tolerance, growth in SM in presence of:</i>	
0.5% NaCl	+
1.0% NaCl	d
2.0% NaCl	—
<i>pH range, growth in SM at pH:</i>	
3.05	—
3.50	d
4.0–7.0	+
7.5	d
8.0	—
<i>Temperature range, growth in SM at:</i>	
30–36°C	+
38°C	d
40°C	—
<i>Ethanol tolerance, growth in SM containing:</i>	
5.5% Ethanol	+
7.7% Ethanol	d
<i>Glucose tolerance, growth in SM containing:</i>	
20% Glucose	+
40% Glucose	d
Growth in SM containing 0.1% neutral red	+
<i>Vitamin requirements:</i>	
Pantothenate and biotin	+
Lipoic acid, folic acid, niacin, <i>p</i> -aminobenzoic acid, riboflavin, cyanocobalamin	—
Catalase	+
Oxidase	—
Acetyl methyl carbinol formed (Voges–Proskauer)	W
Reduction of nitrate	—
H ₂ S produced	d
Survival at 60°C for 5 min	—
Final pH in SM at 30°C	4.8–5.2
<i>Carbon sources:</i>	
D-glucose, D-fructose	+
Sucrose	d
D-Mannose, L-sorbose, D- and L-arabinose, L-rhamnose, D-xylose, D-ribose, D-sorbitol, salicin, dulcitol, D-mannitol, adonitol, erythritol, glycerol, ethanol, D-galacturonate, DL-malate, succinate, pyruvate, DL-lactate, tartrate, citrate, starch, dextrin, raffinose, D-trehalose, maltose, lactose, D-cellobiose	—
Urease	d
Methylene blue reduction	+
Thionin reduction	+
Triphenyltetrazolium reduction	+
Indole	—
Hydrolysis of gelatin, Tween 60, Tween 80	—
L-ornithine, L-arginine, and L-lysine decarboxylases	d
<i>Antimicrobial agents (amount per disk):</i>	
Chloramphenicol, 30 µg; fusidic acid, 10 µg; sulfafurazole, 500 µg; tetracycline, 10 µg	S
Ampicillin, 10 µg; cephaloridine, 10 µg; erythromycin, 10 µg; vancomycin, 10 µg	d
Bacitracin, 5 U; gentamicin, 10 µg; kanamycin, 10 µg; lincomycin, 10 µg; methicillin, 10 µg; nalidixic acid, 30 µg; neomycin, 10 µg; novobiocin, 30 µg; penicillin, 5 U; polymyxin, 300 U; streptomycin, 10 µg	R
Actidione, 0.01%	R

^aFor symbols see standard definitions; R, resistant; S, susceptible; and W, weak.

See Table BXII.α.100 for differentiation of this subspecies from the subsp. *pomacii*.

Isolated from bees, from ripening honey in Spain, from the fermenting sap of *Agave americana* (*atrovirens*) in Mexico, from fermenting palm juice (*Arenga pinnata*) in Indonesia, from sugarcane juice in Queensland, Australia, and the Fiji Islands (Warr et al., 1984), from *Elaeis guineensis* and *Raphia uinifera* in Zaire and Nigeria, and from fermenting sugarcane juice or molasses in Brazil. It has also been isolated in England from beer, from the surface of brewery yards, and from the brushes of cask-washing machines.

The mol% G + C of the DNA is: 47.5–49.5 (*T_m*).

Type strain: ATCC 10988, DSMZ 424.

Additional Remarks: phenotypic centrotypic ATCC 29191.

- b. *Zymomonas mobilis* subsp. ***pomacii*** (Millis 1956) De Ley and Swings 1976, 156^{AL} (*Zymomonas anaerobia* subsp. *pomaceae* (sic) Millis 1956, 527; *Zymomonas mobilis* subsp. *pomaceae* (sic) (Millis 1956); De Ley and Swings 1976, 156.) *pom.* *a' ci. i.* V.L. n. *pomacium* cider; M.V.L. gen. n. *pomacii* of cider.

See Table BXII.α.100 for differentiation of this subspecies from the subspecies *mobilis*.

Isolated in England from sick cider and from apple pulp.

The mol% G + C of the DNA is: unknown.

Type strain: ATCC 29192, NCIB 11200.

Reference strains NCIB 8777 and NCIB 10565.

TABLE BXII.α.100. Differentiation between subspecies of *Zymomonas mobilis*^a

Characteristics	<i>Z. mobilis</i> subsp. <i>mobilis</i>	<i>Z. mobilis</i> subsp. <i>pomacii</i>
<i>Colony diameter after aerobic growth on SM for 7 d at 30°C</i>	1.5 mm	<1.0 mm
<i>Growth in SM at 36°C</i>	+	—
<i>Percent DNA–DNA similarity with strain 5.3^b</i>	76–100	<32
<i>Clustering level of protein electropherograms^c</i>	Cluster together above <i>r</i> = 0.88	Cluster at <i>r</i> = 0.75 with subsp. <i>mobilis</i>
<i>Infrared spectra of intact cells:^d</i>		
Distinct peak at 960 cm ^{−1}	+	—
Shoulder only, 960 cm ^{−1}	—	+

^aSymbols: +, typically positive; —, typically negative.

^bData from Swings and De Ley (1975).

^cData from Swings et al. (1976).

^dData from Swings and Van Pee (1977).

Order V. **Caulobacterales** Henrici and Johnson 1935b, 4^{AL}

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Cau'lo.bac.ter.a'les. M.L. masc. n. *Caulobacter* type genus of the order; *-ales* ending to denote order; M.L. fem. n. *Caulobacterales* the *Caulobacter* order.

The order *Caulobacterales* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA gene sequences; the order contains the family *Caulobacteraceae*.

Description is the same as for the family *Caulobacteraceae*.

Type genus: Caulobacter Henrici and Johnson 1935b, 83 emend. Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1070.

Family I. **Caulobacteraceae** Henrici and Johnson 1935b, 4^{AL}

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Cau'lo.bac.ter.a'ce.ae. M.L. masc. n. *Caulobacter* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Caulobacteraceae* the *Caulobacter* family.

The family *Caulobacteraceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA gene sequences; the family contains the genera *Caulobacter* (type genus), *Asticacaulis*, *Brevundimonas*, and *Phenylobacterium*.

Oligotrophic and chemoorganotrophic, with a strictly aerobic respiratory metabolism. *Caulobacter*, *Asticacaulis*, and some spe-

cies of *Brevundimonas* produce prosthecae; *Phenylobacterium* does not.

Type genus: Caulobacter Henrici and Johnson 1935b, 83 emend. Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1070.

Genus I. Caulobacter Henrici and Johnson 1935b, 83^{AL} emend. Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1070

JEANNE S. POINDEXTER

Cau'lo.bac' ter. L. n. *caulis* stalk; M.L. masc. *bacter* form of Gr. neut. n. *bactrum* rod; M.L. masc. n. *Caulobacter* stalk(ed) rod.

Cells single, unbranched, with tapered poles; long axis typically distinctly curved, conferring a **vibrioid** shape, but may be straight in some isolates and the cell shape **fusiform**; $0.4\text{--}0.6 \times 1\text{--}2 \mu\text{m}$ during growth in most media. The younger pole of the dividing cell bears a **single flagellum**, and the older pole bears a prostheca (the **stalk**) developed by outgrowth of the cell envelope. The stalk includes outer membrane, peptidoglycan, cell membrane, and a core sometimes observed to be occupied in part by membranes but not in any case by any other discernible cytoplasmic components. Stalk diameter is constant along its length, varying from 0.11 to $0.18 \mu\text{m}$ among isolates; the stalk possesses at least one **stalk band** as a substructure after the cell has matured and completed at least one cell cycle. Binary fission is constrictive and is completed without formation of a septum. The stalk-bearing progeny cell grows and eventually repeats the **asymmetric cell division**. The flagellum-bearing progeny cell, after a period of motility, releases the flagellum and develops its stalk at the previously flagellated site as it grows and proceeds to its **asymmetric cell division**. At the base of the flagellum and at the outer tip of the stalk is a small mass of adhesive material, the **holdfast**, which confers adhesiveness on each of the progeny.

Gram negative. Strictly **respiratory** and **aerobic**; O_2 serves as the only terminal electron acceptor for growth, although nitrate may be reduced to nitrite. The principal respiratory quinone is ubiquinone-10. Colonies are circular, convex, and glistening, with smooth margins; butyrous in texture; may be colorless or yellow; upon aging, colorless colonies tend to become light brown. In static liquid cultures, cells accumulate as a **surface film** or pellicle and develop as a ring of growth on the vessel wall at or just below

the air-liquid interface. Growth in agitated liquid cultures is evenly dispersed.

Chemoorganotrophic and **oligotrophic**; grow optimally in media such as peptone-yeast extract containing $0.05\text{--}0.3\%$ (w/v) organic solutes, but not in standard nutrient broth with 0.8% (w/v) organic solutes or richer media such as tryptic soy broth. Do not produce acid or gas from sugars during growth. Optimal temperature for growth $25\text{--}30^\circ\text{C}$; tolerated range for growth $10\text{--}35^\circ\text{C}$. Optimal pH near neutrality; pH $6\text{--}9$ tolerated. Typically do not grow in media containing 2% (w/v) NaCl. Maximum specific rates of exponential growth $0.17\text{--}0.46 \text{ h}^{-1}$. Isolates are diverse in their nutritional requirements. Isolates of one species grow in defined media with glucose or glutamic acid and minerals, other isolates are distinctly stimulated by riboflavin or cyanocobalamin, and some require unidentified growth factors available in peptone. Glucose and glutamic acid are the most widely utilized carbon sources.

Sequences of 16S rDNA are consistent with placement of *Caulobacter* among the *Alphaproteobacteria*. The positions of the 44 sequences grouped as *Caulobacter* in Fig. BXII.α.111 (see "Taxonomic Comments") are represented in Fig. BXII.α.112 by 12 nonredundant sequences which are grouped in Fig. BXII.α.113 and in Fig. BXII.α.114; their strain identities and GenBank accession numbers are displayed in Fig. BXII.α.112. These 12 sequences represent 11 isolates known to exhibit *Caulobacter* phenotypes as well as *Mycoplana segnis*, which is phenotypically distinguishable from *Caulobacter* species. (See "Taxonomic Comments" for descriptions of these trees and for further comments.)

Sources: isolated from distilled, tap and commercial bottled

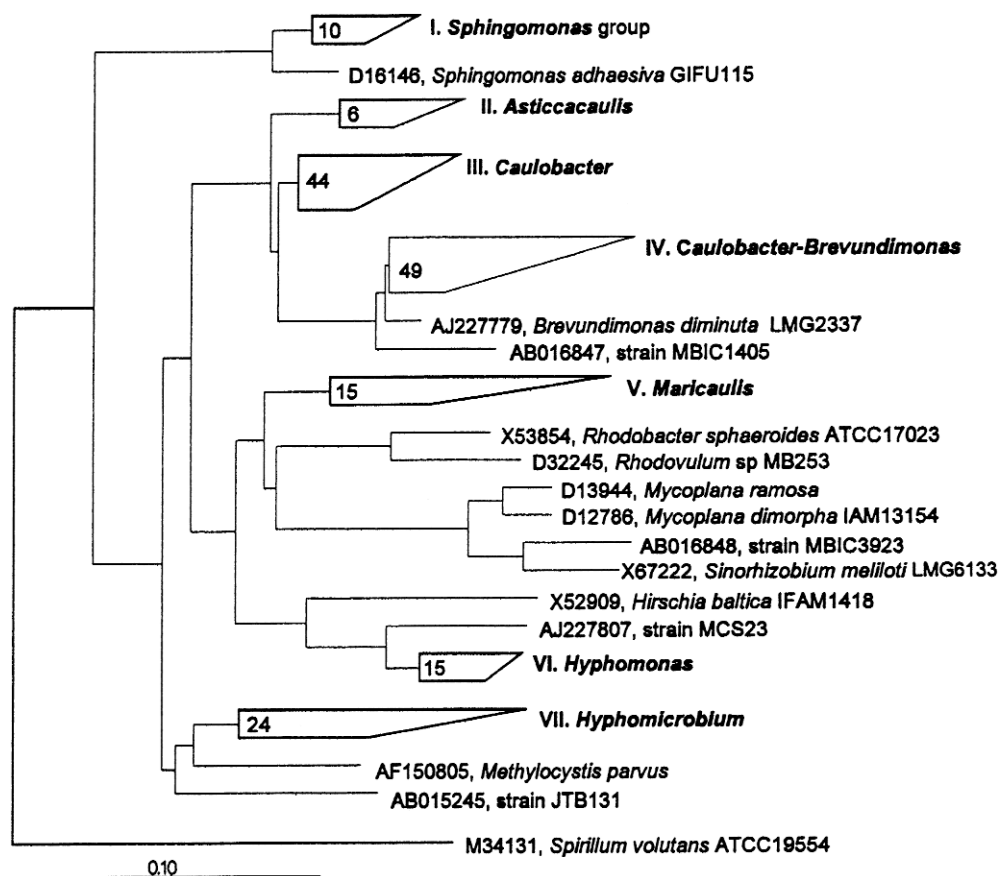


FIGURE BXII.α.111. Phylogenetic tree of 177 16S rDNA sequences built by neighbor-joining analysis, using all available sequences for caulobacters and nonphototrophic hyphal-budding bacteria available as of January 2001. The set includes many sequences that are redundant (determined for the same strain and identical within experimental error), as well as four whose labels are suspect (see text). Within the tree are 99 sequences for caulobacters, 40 for non-phototrophic hyphal-budding bacteria, 13 included by virtue of sequence similarity without phenotypic descriptions of the isolates, and 25 for isolates that are phenotypically distinguishable from prosthecate bacteria. The seven groups accommodate all but 14 of the sequences; excluded are one caulobacter (strain MCS23), one hyphal-budding bacterium (*Hirschia baltica* IFAM1418), three sequences for undescribed organisms, and nine for isolates known to be phenotypically distinguishable from caulobacters and hyphal-budding bacteria. Bar indicates evolutionary distance. Each enclosed numeral indicates the number of sequences grouped. The minimum similarities within each of the caulobacterial genus groups are: I. *Sphingomonas* group (*C. leidyi*), 95.7%; II. *Asticcacaulis*, 95.9%; III. *Caulobacter*, 94.9%; IV. Group IV caulobacters, 94.8%; and V. *Maricaulis*, 94.7%. See Fig. BXII.α.114 for a more stringent (maximum-likelihood) analysis of these sequences.

drinking water, natural bodies of fresh water, and sewage (principally from activated sludge during secondary treatment). Not encountered as clinical isolates and not known to be pathogenic for plants or animals.

The mol% G + C of the DNA is: 62–65.

Type species: *Caulobacter vibrioides* Henrici and Johnson 1935b, 84.

FURTHER DESCRIPTIVE INFORMATION

Morphology Morphological details of *Caulobacter* cells are illustrated in Fig. BXII.α.115, Fig. BXII.α.116, and Fig. BXII.α.117. Cell shape and life cycle are major characteristics in the description of caulobacters, and the redistribution of these bacteria among five genera (see “Taxonomic Comments”) increases the usefulness of morphology in the description of each genus. Cells of *Caulobacter* isolates have distinctly tapered poles; only isolates of the species *C. fusiformis*, atypical in several ways, yet more reasonably accommodated here than elsewhere in the

family, lack the distinct curvature of the others, all of which are vibrioid. Neither the sharp tapering nor the cell curvature varies with cultivation in liquid media under conditions that support exponential growth of morphologically uniform populations. Morphology of these, as of most bacteria, becomes aberrant when cells are subjected to conditions unfavorable for growth. In unfavorable conditions (e.g., being buried under millions of other cells in a colony on an agar surface, or in media rich in soluble organic nutrients, ammonium ions, or NaCl), cells may become irregular in many ways, which include branching of cells or of stalks, uneven bloating, failure of stalk elongation, development of supernumerary stalks, loss of motility, heavy shedding of the S-layer and other cell surface components, frequent lysis and, typically, a decrease in ability to form colonies, i.e., loss of viability. Such populations are not suitable for description of the morphology of an isolate.

Morphology is uniform, swimmers are motile, cells are adhesive, stalks elongate, and bands are added through successive

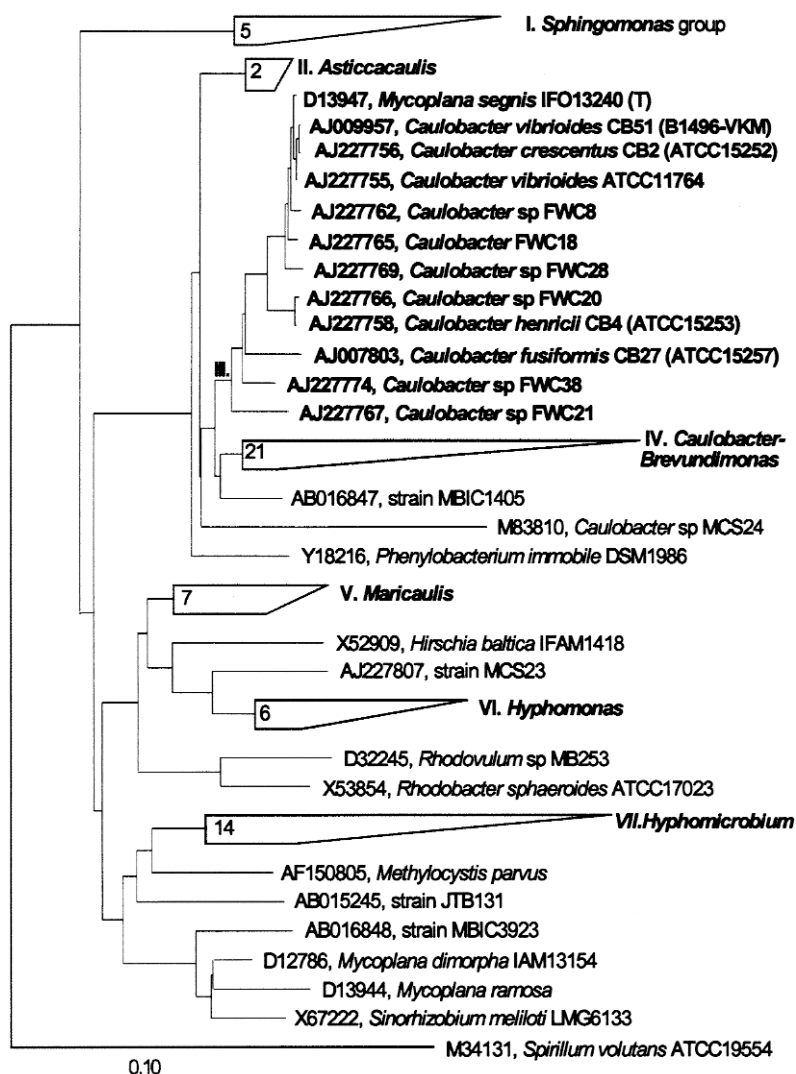


FIGURE BXII.α.112. Phylogenetic tree of 81 16S rDNA sequences, with group III. *Caulobacter* expanded to display GenBank accession numbers and strain identities. (See Fig. BXII.α.114.) Also represented are the positions of sequences AB023427 (by D13947); AB008531, AF125194, AJ227754, AJ227757, AJ227768, M83798, M83799, M83801, M83802, M83803, M83804, and X52281 (by AJ009957 and AJ227756); AJ227761, AJ227763, AJ227764, AJ227773, AJ227776, and AJ227777 (by AJ227755); AJ227770 (by AJ227762); AJ227772 and AJ227775 (by AJ227765); AJ227760 and AJ227771 (by AJ227769); AB008532 and AJ007805 (by AJ227758 and AJ227766); AB008533, AJ227759, and M83796 (misidentified as *Caulobacter bacteroides* strain CB7) (by AJ007803); and M83805 (by AJ227774). Bar indicates evolutionary distance. Each enclosed numeral indicates the number of sequences grouped.

cell cycles when *Caulobacter* isolates are cultivated aerobically in dilute media at neutral pH and at or below 30°C. Isolates identifiable as *Caulobacter* species by additional, non-morphological criteria fail to thrive and typically die in rich media, apparently due to inhibition of one or more steps in their morphogenetic sequence. "Rich" may apply to a single excess nutrient, as demonstrated for ammonium by Felzenberg et al. (1996). Generally, for any isolate, mean stalk length decreases with increasing concentration of nutrients in peptone-yeast extract media and with increasing phosphate concentration in both complex and defined media (Schmidt and Stanier, 1966; Haars and Schmidt, 1974; Poindexter, 1984; Felzenberg et al., 1996; Poindexter and Staley, 1996). Growth of the majority of isolates is adversely affected by phosphate concentrations in excess of 5 mM.

The flagella of most species contain three flagellins of slightly

different mobilities (29, 27, and 25 kDa) in denaturing electrophoretic gels. The periodic synthesis and assembly of the flagellum of *C. crescentus* and of chemotaxis proteins are influenced by a hierarchy of expression of dozens of genes that is coordinated with the regulation of many other growth and morphogenetic genes (reviewed in, *inter alia*, Wu and Newton, 1997). The basal body has five rings, rather than the four typical of Gram-negative cells; it is possible that the fifth ring participates in some way in the regular shedding of the *Caulobacter* flagellum that precedes the onset of stalk development and general cellular growth.

Fine structure Stalk bands (see Figs. BXII.α.115D, BXII.α.116C, and BXII.α.117C) occur in all *Caulobacter* isolates. Each band is a set of concentric rings that are more electron-opaque and structurally more rigid than the remainder of the

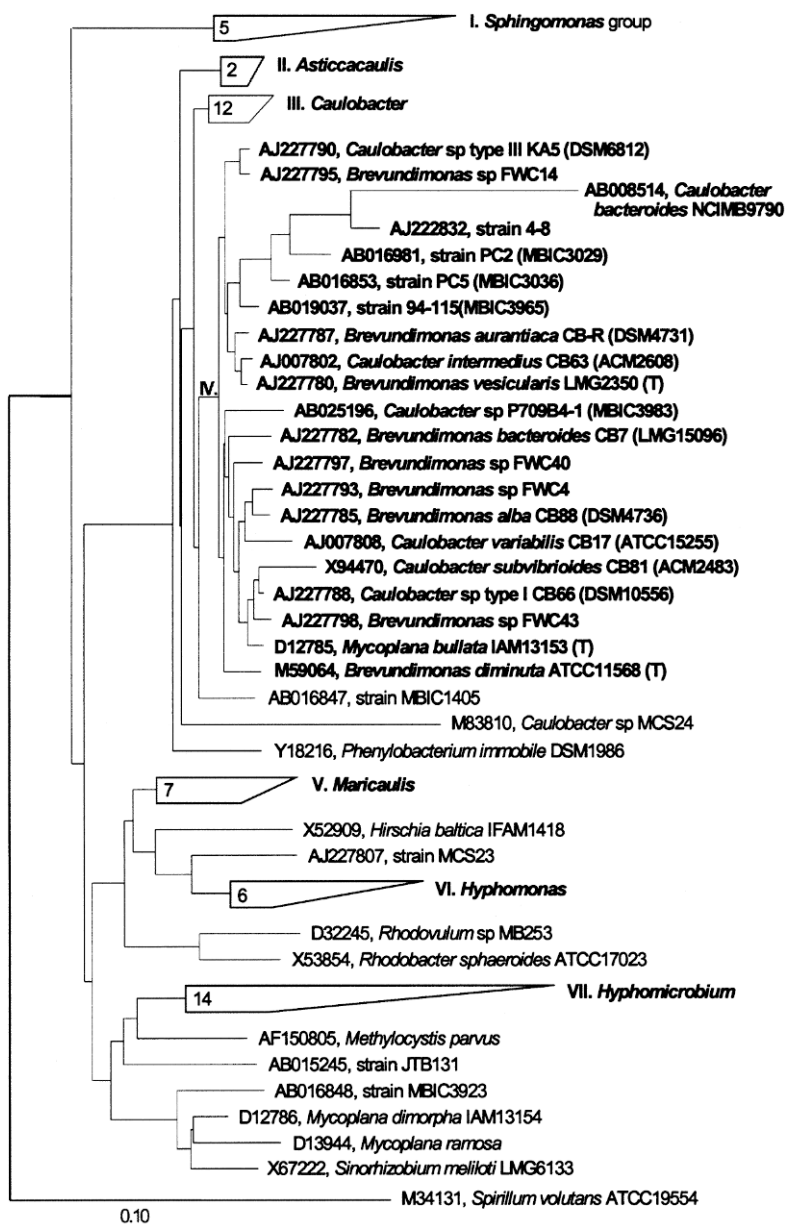


FIGURE BXII.α.113. Phylogenetic tree of 81 16S rDNA sequences, with group IV caulobacters expanded to display GenBank accession numbers and strain identities. (See Fig. BXII.α.114) Also represented are the positions of sequences M83800 (by AJ227795); AB016980 (by AB016981); AB016846, AB016849, AB016850, AB016851, AB016852, and AJ227796 (by AJ227787); AB023784 (by AJ007802); AB021414, AJ007801, and AJ227781 (by AJ227780); AJ227799 and M83808 (by AB025196) (neighbor-joining analysis groups M83810 with these sequences, although maximum-likelihood analysis places it in a unique position); AB008513 and AJ227792 (by AJ227782); AJ227794 (by AJ227793); AB008393 (by AJ227785); AB0083392 and AJ227784 (by X94470); AJ227789 and AJ227791 (by AJ227788). The position of the *Mycoplana bullata* sequence AB023428 is represented by D12785, same species. The positions of *Brevundimonas diminuta* sequences AB021415, AJ227778, and X87274 are represented by M59064, same species. Two additional sequences available for *B. diminuta* are not represented: D49422 falls within this group, but at a unique position, and AJ227779 falls outside the group (see Fig. BXII.α.111). Bar indicates evolutionary distance. Each enclosed numeral indicates the number of sequences grouped.

stalk (Jones and Schmidt, 1973). Earlier evidence that one band is added to the growing stalk during each cell cycle completed by the mature stalked cell of *C. crescentus* (Staley and Jordan, 1973) has been confirmed (Poindexter and Staley, 1996) and this trait exploited in the determination of the reproductive rate of diverse caulobacters *in situ* in a freshwater lake (Poindexter et al., 2000). The stalk band appears to be one more cellular

feature to be added to the growing list of cell cycle-regulated events in the life of *C. crescentus*, and presumably of other species as well.

The cell and stalk of *C. crescentus* have long been known to be coated with an S-layer (Poindexter et al., 1967), a cellular feature of a wide variety of both Gram-negative and -positive bacteria (Sleytr et al., 1988). J. Smit and his coworkers have dem-

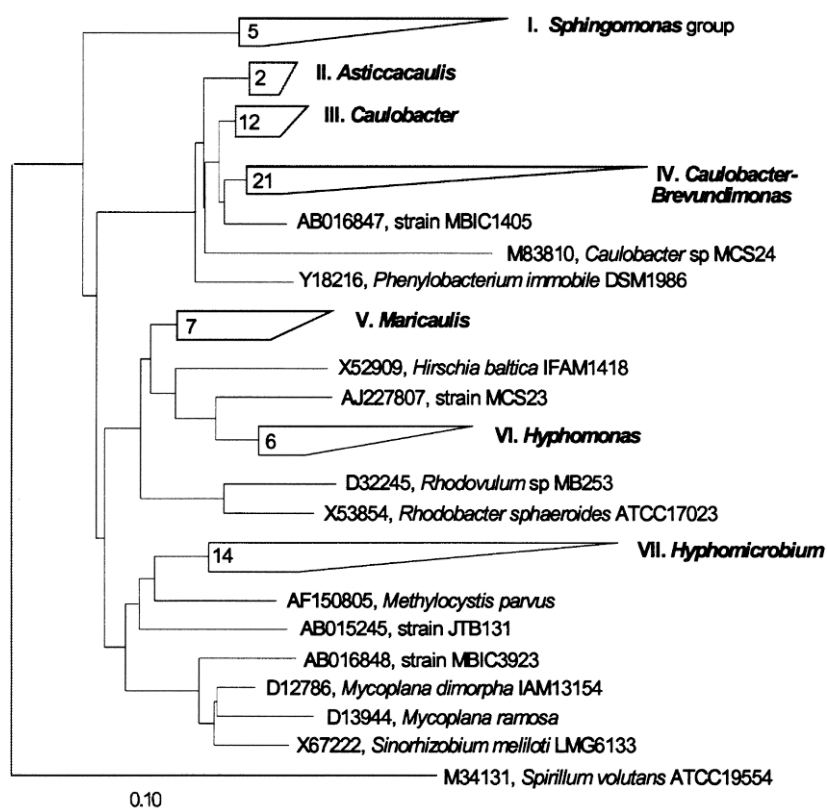


FIGURE BXII.α.114. Phylogenetic tree of 98 16S rDNA sequences built by maximum likelihood (DeSoete) analysis. After removal of redundant sequences (determined to be for the same strain and identical within experimental error), the four sequences whose labels are suspect, and one sequence that appeared to be only very distantly related (X97079, strain TM16; not described phenotypically), all positions of the complete tree are here represented by 81 sequences: 37 for caulobacters, 20 for hyphal-budding bacteria, 14 for bacteria phenotypically distinguishable as nonprosthecate bacteria, and 10 included by virtue of sequence similarity without phenotypic descriptions of the isolates. Relevant branches are expanded in Fig. BXII.α.112 and BXII.α.113 and in other chapters (*Asticcacaulis*, Fig. BXII.α.119 and *Maricaulis* Fig. BXII.α.76), where GenBank accession numbers and strain identities are shown, and sequences represented but not shown are listed in the legends. Bar indicates evolutionary distance. Each enclosed numeral indicates the number of sequences grouped.

onstrated that this structural feature is typical of *Caulobacter* isolates; exceptions occur only among isolates that are atypical in other respects as well. Attachment of the S-layer to the cell surface is mediated by calcium and requires an anchoring oligosaccharide. Both the S-layer protein and the oligosaccharide are antigenic, and serological cross-reaction occurs among the S-layers and their anchors (Walker et al., 1992). An S-layer gene probe prepared from *C. crescentus* detects similar genes in most *Caulobacter* isolates, but not in Group IV caulobacters or random nonprosthecate isolates from wastewater samples that yielded *Caulobacter* spp., suggesting one cultivation-independent means for enumeration of *Caulobacter* spp. in environmental samples (MacRae and Smit, 1991). Although an S-layer may provide some defense against *Bdellovibrio* predation (Koval and Hynes, 1991), the function of this layer is as uncertain in *Caulobacter* species as it is in other bacteria.

A third surface structure of all caulobacters is the holdfast. Again, it is J. Smit and his coworkers who have most thoroughly examined this structure, both in regard to its chemistry (Merker and Smit, 1988; MacRae and Smit, 1991; Abraham et al., 1999) and (in *C. crescentus*) its genetics (Mitchell and Smit, 1990). As noted by Merker and Smit (1988), the function of this organelle is uncomplicated in caulobacters; in nature, it serves to mediate

adhesion of the cell to surfaces. It does not coat the entire cell surface, trap molecules, or join cells in biofilms. In dense laboratory cultures, cells stick to each other in rosettes; while that is helpful in recognition and differentiation of caulobacter isolates by microscopy, it is an artifact of cultivation (Poindexter, 1964). Some *Caulobacter* species also bear pili, which typically arise around the base of the flagellum and disappear as stalk development is initiated. Pili probably also participate in adhesion of cells to surfaces, and are demonstrably able to bind RNA bacteriophages (Schmidt, 1966).

Assays based on the binding of plant lectins by holdfasts, the susceptibility of adhesiveness to competition by monomers, dimers and trimers of *N*-acetylglucosamine, and the stability of adhesion in the presence of various hydrolytic enzymes have consistently implied that the holdfasts of most *Caulobacter*, Group IV caulobacters, and *Maricaulis* (but not *C. leidy*) isolates consist of or contain some oligo-*N*-acetylglucosamine that participates in adhesion. In contrast to the marine caulobacters, all of which bind wheat-germ agglutinin (WGA) on their holdfasts, some isolates of each of the freshwater genera did not bind WGA; some of those also failed to bind any of six other lectins tested, indicating some diversity of holdfast composition in the freshwater genera (Merker and Smit, 1988).

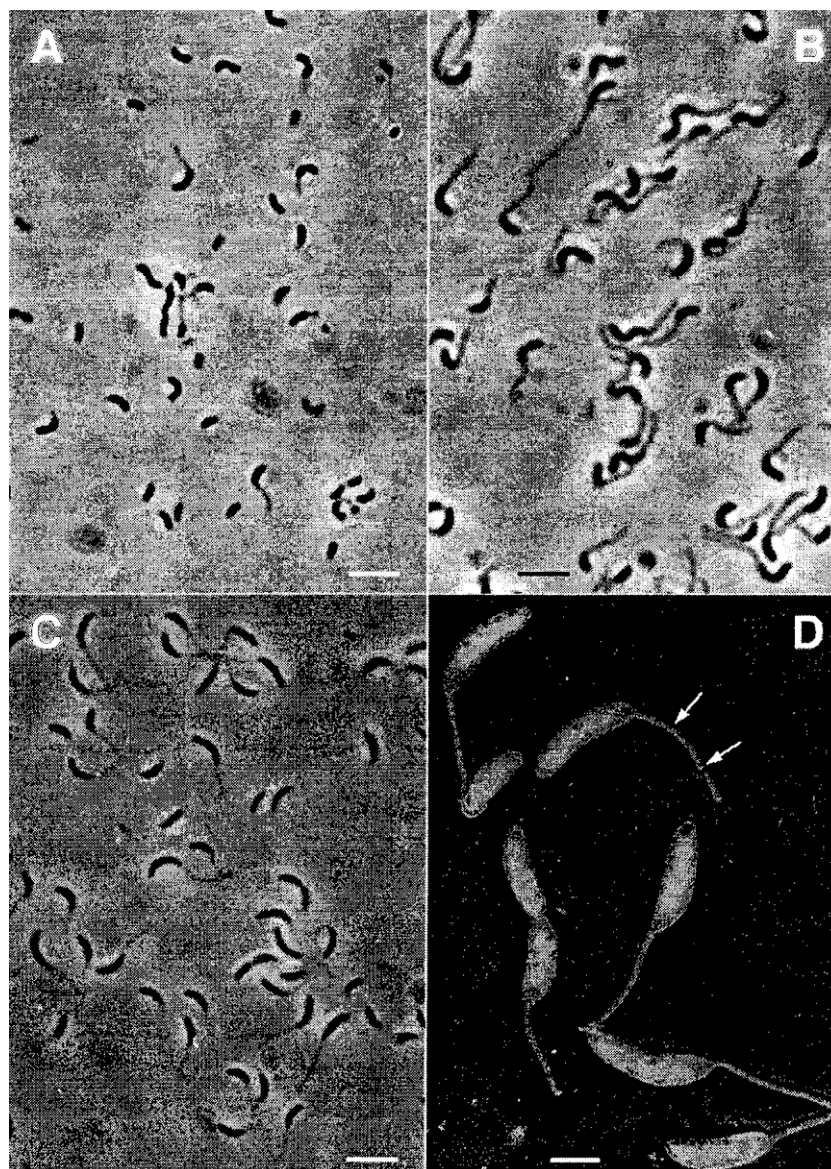


FIGURE BXII.α.115. *C. crescentus* grown in peptone–yeast extract medium (A) and in glucose–glutamate minimal medium (B–D). A–B, strain CB15 (ATCC19089), phase-contrast microscopy of dried smears to which Gray's flagella stain mordant and cover slips were added. C–D, strain CB2 (ATCC15252). C, phase-contrast microscopy, wet mount. D, electron microscopy, shadowed specimen, negative image; arrows indicate stalk bands. Bars = 5 μm (A–C) and 1 μm (D).

Cellular composition All isolates examined possess a thin peptidoglycan sacculus that extends continuously through the cell pole into the stalk. The peptidoglycan is composed of essentially the same directly cross-linked, diaminopimelic acid-containing polymer that is typical of Gram-negative bacteria. However, while whole-cell peptidoglycan exhibits a 50% excess of muramic acid over glucosamine (Poindexter and Hagenzieker, 1982), the relative proportions of the two sugars appear to be reversed in the stalk peptidoglycan, which has been assayed separately only for *C. crescentus*. This implies that stalk outgrowth involves a modification of the peptidoglycan or of its synthesis. The composition of the peptide side chains of the peptidoglycan may vary with medium composition; glycine is a component in some media (Markiewicz et al., 1983), but it is not invariably

detected in the peptidoglycan. Major membrane proteins unique to the stalk have not been discerned.

The principal phospholipid in *Caulobacter* and group IV caulobacter cells is phosphatidylglycerol, and the most abundant fatty acid species are $\text{C}_{18:1}$ and $\text{C}_{16:0}$. All isolates tested also contain lower, but significant amounts of $\text{C}_{18:1} \omega 7 \text{c}$ 11CH_3 and of species of $\text{C}_{17:1}$, $\text{C}_{16:1}$, $\text{C}_{17:0}$, $\text{C}_{15:0}$, $\text{C}_{14:0}$. *Caulobacter* isolates contain $\text{C}_{12:1} \text{3OH}$ and an irresolvable mixture of C_{12} fatty acids designated ECL 11.798 by Abraham et al. (1999). A single type of phosphoglycerolipid (PGL), 3-O-[6'-(*sn*-glycero-3'-phosphoryl)- α -D-glycopyranosyl]-*sn*-glycerol, is identifiable in this genus. This is lipid pattern C of Abraham et al. (1997), found in 34 of 37 *Caulobacter* isolates, but in only one of 19 Group IV caulobacter isolates tested. The three exceptional *Caulobacter* strains contained PGLs

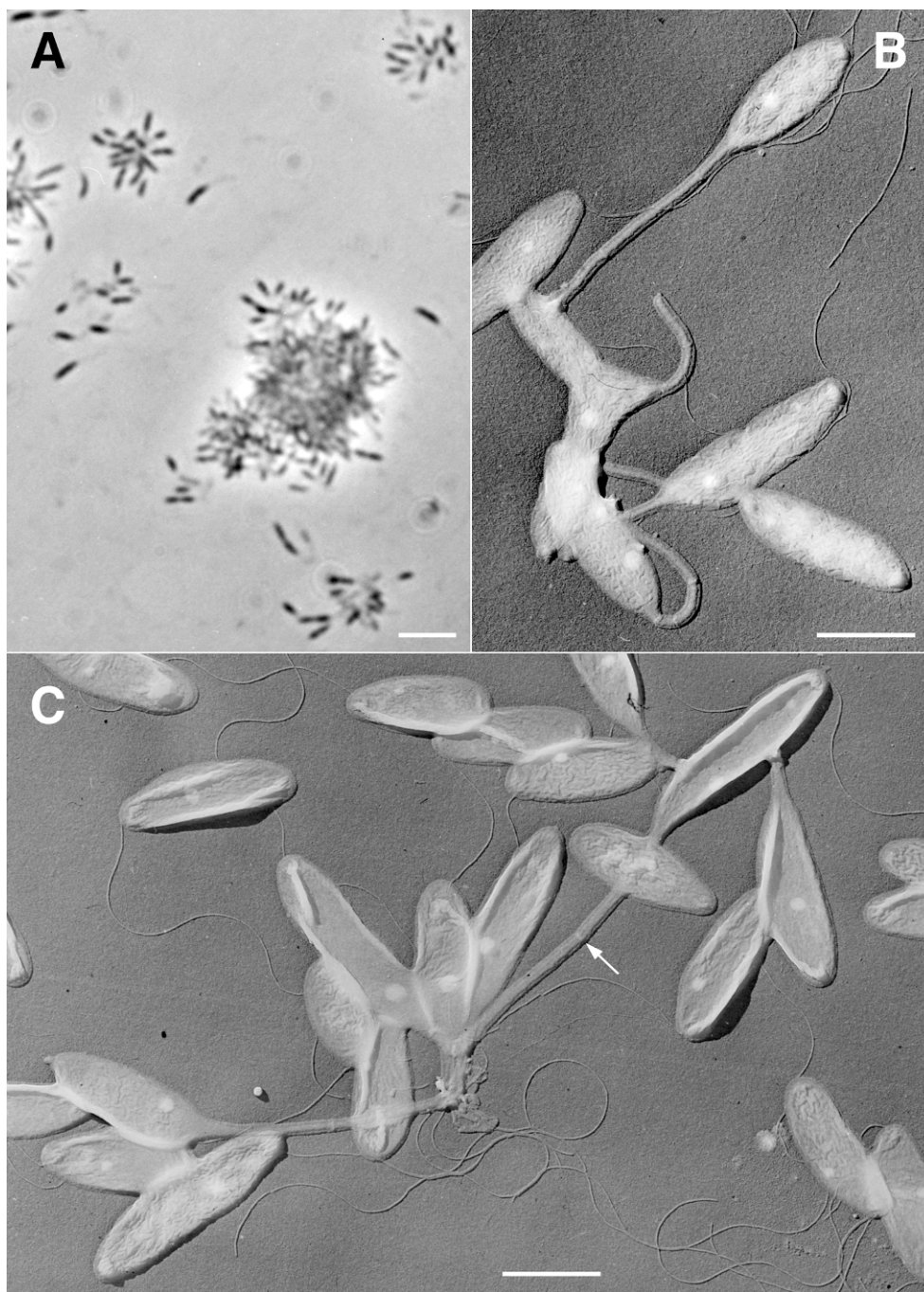


FIGURE BXII.α.116. *C. fusiformis* strain CB27 (ATCC15257) grown in peptone–yeast extract medium. A, phase-contrast microscopy, wet mount. Bar = 5 μm . B–C, electron microscopy, shadowed specimen, negative image. Arrow in (C) indicates a stalk band. Y-shaped cells, like the one visible here, are not common. Bars = 1 μm .

that also occur in some Group IV caulobacter isolates. Typically, Group IV caulobacters are distinguished from *Caulobacter* by the presence of $\text{C}_{12:0\ 3\text{OH}}$ and the absence of $\text{C}_{12:1\ 3\text{OH}}$ and ECL 11.798. Eight of 12 Group IV caulobacter isolates analyzed contain the PGL typical of *Caulobacter*, and all 12 also contain at least two other PGL types resolvable by the chromatographic methods used in these analyses. This is lipid pattern B of Abraham et al. (1997), found in 18 of 19 Group IV caulobacter isolates, but in only two of 37 *Caulobacter* isolates tested. Five of 12 Group IV caulobacter isolates also contain sulfoquinovosyl diacylglycerols,

one of which was identified as 1-O(α -6',6-deoxy-aldohecopyranosyl-6'-sulfonic acid)-3-O-diacylglycerol. Such compounds are uncommon in nonphototrophic bacteria, but are typical of *Mari-caulis* isolates.

Enzymes As far as tested, *Caulobacter* and Group IV caulobacters employ the Entner–Doudoroff pathway for hexose catabolism. Enzymes for catabolism of some carbohydrates are induced by exposure to substrate, but induced levels are not much more than 10-fold higher than noninduced levels (Poindexter,

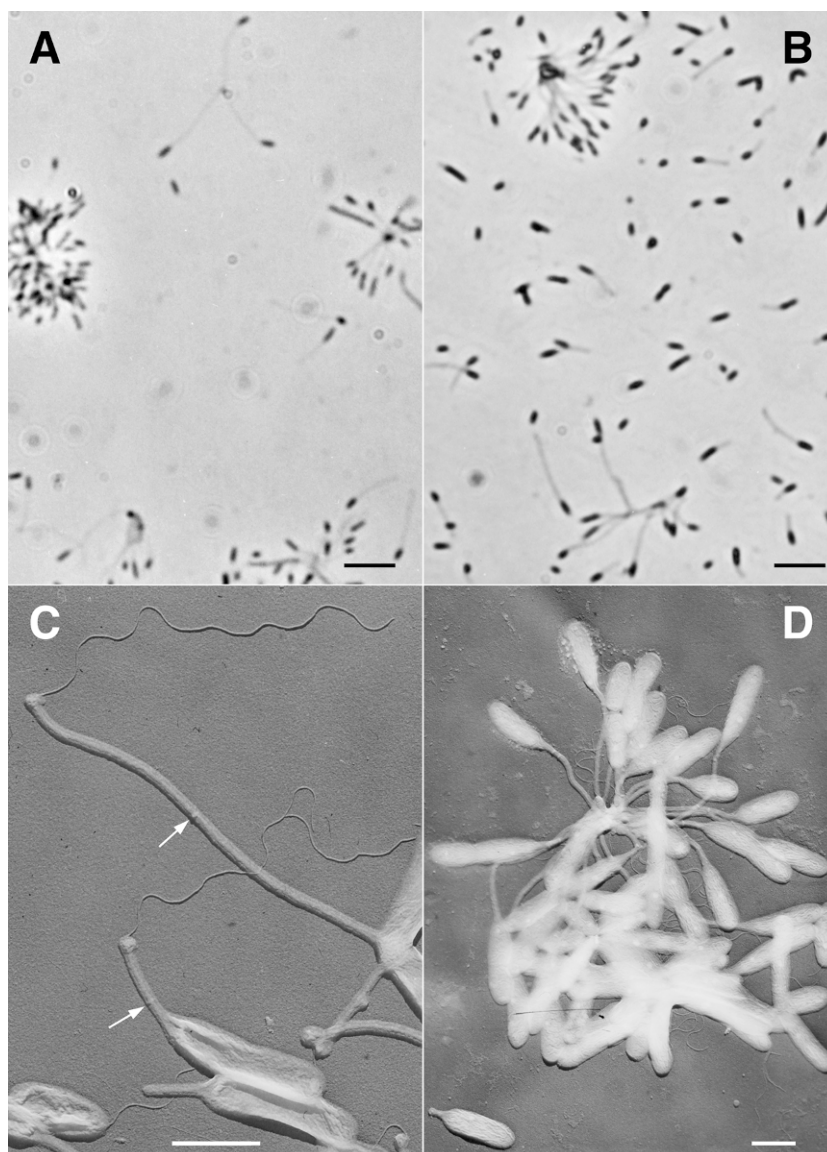


FIGURE BXII.α.117. Group IV caulobacter isolates of the bacteroides cell shape grown in peptone–yeast extract medium. A–C, strain 25 (VKM v-1183); D, strain CB11a (ATCC19090). A, phase-contrast microscopy, wet mount. B, phase-contrast microscopy of dried smear to which Gray's flagella stain mordant and cover slip were added. C–D, electron microscopy, shadowed specimens, negative image. Arrows in C indicate stalk bands. A–B, Bars = 5 μ m; C–D, Bars = 1 μ m.

1981). Ammonium assimilation has been examined only in *C. crescentus*, which lacks glutamic dehydrogenase and employs the glutamine synthetase–glutamate synthase system only (Ely et al., 1978). This may in part account for susceptibility of growth to excessive (relative to glutamate) availability of ammonium (Felzenberg et al., 1996). *C. crescentus* cells have more than one enzymatic defense against toxic oxygen species. Two superoxide dismutases (SOD) are made, a cytoplasmic Fe-SOD and a Cu-Zn-SOD that is localized in the periplasm (Steinman and Ely, 1990); the ability of the periplasmic enzyme to protect cells against extracellular superoxide has been demonstrated (Schnell and Steinmann, 1995). *C. crescentus* also produces a catalase/peroxidase whose activity increases significantly in the stationary phase of laboratory cultures (Schnell and Steinmann, 1995). The pres-

ence, location, and inducibility of these enzymes may contribute to two characteristics of caulobacter ecology: their tolerance of close association with O₂ generators such as algae, and their slow deterioration during periods of nutrient deprivation.

Genome structure The complete genome sequence of *Caulobacter crescentus* strain CB15 has been determined (Nierman et al., 2001) and is available through GenBank as accession number AE005673. *C. crescentus* was the first free-living alphaproteobacterium to be analyzed this thoroughly. In the report an interpretation of the genome structure and probable roles of the 3767 genes recognized is presented. The report by Nierman et al., (2001), in particular, gives considerable attention to the characteristics of this genome that are consistent with the oligotrophic, aquatic existence of the organism.

DNA–DNA hybridization has not been used extensively with caulobacters since the study by Moore et al. (1978), where only *C. vibrioides* and *C. crescentus* showed hybridization values greater than 58%, and some pairs of strains were more than 90% homologous. Analysis of restriction endonuclease digests of the 16S–23S rDNA interspacer regions (ISR) of *C. vibrioides* and *C. crescentus* strains and of other *Caulobacter* isolates revealed two patterns, but neither was indicative of either species (Abraham et al., 1999). More such analyses—both hybridization and ISR analysis—would be helpful in determining the extent of genomic overlap between isolates that grow vigorously, rapidly, and independently of riboflavin (*C. crescentus*) and those that grow at lower rates, with lower yields, and require both riboflavin and other unidentified organic micronutrients (*C. vibrioides*).

Plasmids, often quite large (>50 kb), have been detected in some *Caulobacter* and Group IV caulobacter isolates (Gregory and Staley, 1982; Schoenlein and Ely, 1983; MacRae and Smit, 1991). The identities of genes on the native plasmids have not yet been determined. However, expression of several foreign plasmid-borne genes has been demonstrated in *C. crescentus*. These include genes for mosquitocidal toxins from Gram-positive bacteria (Thanabalu et al., 1992), and a phosphate-regulated porin gene from *Pseudomonas aeruginosa* (Walker et al., 1991). Ribosomal rDNA sequences have been determined for a large number of caulobacter isolates. *Caulobacter* sequences constitute one of five coherent groups among caulobacterial sequences. The minimum similarity among sequences grouped within “III. *Caulobacter*” in Fig. BXII.α.111 is 94.9%. All known sequences for 16S rDNA are less similar except sequences for organisms identified as *Mycoplana segnis* (95.9–99.9%) and *Phenylobacterium immobile* (95.5–96.4%). See “Taxonomic Comments,” below, for further discussion of 16S rDNA sequences and of bacteriophages.

Antibiotic susceptibilities *Caulobacter* and Group IV caulobacter isolates obtained from natural fresh water and from drinking water have usually exhibited susceptibility to both cell wall- and protein synthesis-inhibiting antibiotics (Poindexter, 1964; Nikitin et al., 1990; MacRae and Smit, 1991), but resistance has been encountered in the majority of isolates from wastewater treatment systems. Some of those isolates appear resistant to antibiotics of various chemical types, targets, and mechanisms of antibacterial action, implying that sewage treatment facilities—like hospitals—may provide environments that encourage the propagation, and possibly the transfer, of genes for antibiotic resistance (MacRae and Smit, 1991).

ENRICHMENT AND ISOLATION PROCEDURES

Because of their unique morphology, caulobacter cells can be recognized in natural materials by phase-contrast and electron microscopy. Generally, they are most readily observed in water samples with low soluble organic nutrient content and among the bacteria attached to the surfaces of algal thalli. They are commonly found among the bacterial contaminants of unialgal cultures and may influence the course of development in the algae (Klaveness, 1982). They are also frequently detected in moist soils, either attached to diatoms or among members of rhizosphere communities, and in activated sludge. In samples from such environments, particularly aqueous ones, caulobacters may account for more than 40 of every 100 viable aerobic, oligotrophic, chemoheterotrophic bacteria. When they occur in such relative abundance, caulobacters can be isolated by direct

plating, and their colonies identified and selected for purification as described below for colonies from enrichment cultures.

The lack of identification of any physiologic peculiarity within this group has prevented the development of a strongly selective enrichment procedure. Enrichment depends on a mechanical process: the accumulation of stalked cells in the air–water interface of a water sample or soil extract that has been protected during incubation and examination against turbulence, which would disturb the air–water interface. The most dependable, although tedious, procedure for enrichment and isolation of caulobacters has not changed since it was introduced by Houwink (1955). A sample of water or a water extract of soil is enriched with at most 0.01% (w/v) peptone and allowed to stand at room temperature. Within a few days, a sample of the surface film is taken with a bacteriological loop or a cover slip and is examined by phase-contrast microscopy for the presence of stalked cells. Some cells may possess a stalk at each pole and so be indistinguishable from *Prostheco bacter* (*q.v.*) until clones have been isolated and examined for the motility characteristic of caulobacters. When caulobacter cells account for 10–50% of the population, a sample (again, taken with a loop, not a pipette) is streaked on a dilute (0.05%, maximum 0.1%, w/v) peptone or peptone–yeast extract medium prepared with 1.0% or 1.5% agar. By the third or fourth day of incubation, small, hyaline or crystalline, non-iridescent colonies of caulobacters begin to appear. It is helpful to remove these colonies while they are very small, by using sterile toothpicks, to small sites (“patches”) on secondary plates, for two reasons. First, samples of oligotrophic populations often include bacteria that swarm as a continuous film on the agar surface and invade other colonies. Second, preparation of a wet mount to screen the initial, small colony may consume the entire colony. It is preferable to begin microscopic screening only after the patches of growth on the secondary plate have developed.

Identification requires phase-contrast microscopy because of the small diameter of the stalk (<0.2 μ m), which is below the resolution afforded by ordinary light microscopy. The addition of a stain to the wet mount or the use of a mordanted stain such as is designed for flagella may be helpful. The mordant alone applied to dried smears clearly reveals stalks by phase-contrast microscopy. However, examination of wet mounts of living cells is necessary because motility is characteristic of caulobacters, and its detection aids particularly in distinguishing caulobacter and Group IV caulobacters from *Prostheco bacter*. In the dense populations developed on an agar surface, caulobacter cells will adhere to each other’s holdfasts, and the characteristic rosettes with cells peripheral to the stalks, which are united in a common holdfast, are often the most dependable way to detect the presence of stalks. In enrichment samples and in pure populations, stalked cells become trapped within air bubbles in wet mounts, and stalks not clearly discernible in the suspended population are more obvious in such regions.

Typical caulobacters multiply less rapidly than do bacteria such as *Pseudomonas* and *Flavobacterium* species that are often present in the same samples. It has been suggested that serial dilutions of the sample might allow the caulobacters to develop without becoming outnumbered by such other types. However, this procedure is suitable only when the caulobacters are initially predominant and are not attached to other cells, two conditions not commonly met. Particularly in dilute media, the bacteria that develop first exhaust the nutrients and preclude multiplication of slower organisms, even if those were more numerous in the initial sample (Belyaev, 1967; Poindexter, unpublished).

It is also possible to enrich a water sample *in situ* by "baiting" the caulobacters with cover slips, to which they will attach. The submerged cover slip can be removed, rinsed well with sterile water, then used as inoculum for a stationary culture as described above. Caulobacter attachment is tenacious; the cells cannot be efficiently scraped from the cover slip but should be allowed to release swimmers by cell division. Some success with thin plastic foil carefully laid on the surface of quiet water has also been reported.

In any of these procedures, the most helpful factor important to successful isolation is that the sample be low in soluble organic nutrient content. Caulobacters are not necessarily absent from richer samples, but their proportion is typically low, and their reproductive rate is not competitive in nutrient-rich cultures. Nevertheless, they can be isolated from soil (e.g., Poindexter, 1964; Belyaev, 1969) and from wastewater treatment facilities, particularly from secondary-stage activated sludge (MacRae and Smit, 1991).

MAINTENANCE PROCEDURES

Once isolated, *Caulobacter* species can be dependably maintained in at least four ways. First, vegetative stocks should be maintained on 1% agar slants of dilute (0.05–0.3% organic material) complex medium or defined medium (if possible, with a given isolate) that contains at least 100 mg organic carbon/mg phosphate-phosphorus and 1 mM each of MgSO_4 and CaCl_2 . Such a medium promotes the storage of carbon reserves and prolongs the viability of the population during storage. Isolates should be transferred every 8 or 9 weeks, incubated 2 or 3 days at 20–25°C (higher incubation temperatures reduce stability during storage), then refrigerated. Second, cells grown in dilute complex medium can be stored in small volumes frozen at –70°C without cryoprotectant. Survival varies among isolates, ranging from 10 to 50%. Such frozen cultures can be thawed at room temperature and transferred to growth medium to resume vegetative cultivation. This is the most dependable method of maintenance of freshwater and soil caulobacters. Third, washed cells diluted to 10^3 – 10^4 cells/ml of sterile water and sealed in ampules can be stored at room temperature for 2–3 years. This procedure is more dependable than lyophilization but requires periodic recultivation. The fourth method, lyophilization, is the least dependable means of maintenance. If a protectant such as milk solids is used, the rehydrated specimen should be diluted immediately in order to avoid the inhibitory effect of the solids. Lyophilization on strips of sterile filter paper, without any additive, has proven to be the most dependable means of lyophilization.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

All caulobacter species groups are distinguished by the morphology of the dividing cell—specifically, by the asymmetry of its appendages. Both appendages are best characterized by electron microscopy. Shadowed specimens provide the clearest image, but negatively stained specimens need not be washed and so preserve a higher proportion of attached flagella. The holdfast is best detected by its adhesive function, although it, too, is discernible by electron microscopy.

DIFFERENTIATION OF THE GENUS *CAULOBACTER* FROM OTHER GENERA

Three groups of bacteria other than caulobacters produce long cellular appendages of the envelope that are of constant diameter. These bacteria may occur in habitats with caulobacters, and a motile stage is produced by one type (Poindexter, 1992). Nev-

ertheless, they are distinguishable from caulobacters, and from each other, by the morphology of their reproductive stages, as shown in Table BXII.α.101. Relationships to non-prosthecate bacteria implied by 16S rDNA analysis are illustrated and discussed under "Taxonomic Comments," below. The five species groups of caulobacters are distinguished as shown in Table BXII.α.102.

TAXONOMIC COMMENTS

The genus *Caulobacter* was created by Henrici and Johnson (1935b) on the basis of their observations of bacterial populations attached to slides submerged in a freshwater lake. They did not obtain isolates or study pure cultures. Nevertheless, they correctly inferred the developmental sequence of the life cycle and recognized that this type of bacterium was unique. The laboratories of several investigators, notably Shapiro, Newton, Brün, Mullins, Gomes, Marczyński, Gober, and Ely, continue to explore that life cycle and the intricate regulatory genetic system that guides it (Gober and Marques, 1995; Marczyński and Shapiro, 1995; Wu and Newton, 1997). Those genetic and developmental studies have exploited the ability of the species *C. crescentus* to grow in unsupplemented, chemically defined media and thereby to present itself as the caulobacter most suitable for detectable expression of genetic change. *C. crescentus* has been found to be amenable to various means of genetic manipulation and analysis and is now one of the most thoroughly characterized bacterial genomic systems. Focus on that species has yielded a vast amount of information with considerable significance for prokaryotic developmental biology and molecular genetics. The major taxonomic import of those studies is the finding that the type of asymmetric cell division unique to the caulobacters reflects a complex of genes, not a single gene or operon or cassette, whose expression has been interwoven into the information that guides reproduction and on which viability depends. Whatever the non-prosthecate ancestry of this group, evolution of the caulobacter type of life cycle has clearly involved steps that led to a nearly irreversible commitment to it. The rarest morphogenetic mutant of *C. crescentus* is a stalkless but otherwise unaltered clone, although loss of motility or of adhesiveness can occur through mutations that do not affect other aspects of morphogenesis or viability. Nature may yet provide a recognizable stalkless caulobacter, but at first isolation, it is certain to be assigned to a non-prosthecate taxon. Meanwhile, nature does provide a diversity of bacterial types that exhibit the life cycle inferred by Henrici and Johnson. That diversity is the focus of this and two other chapters (*Asticcacaulis* and *Maricaulis*) in this *Manual*. These comments are related to all three chapters and to all four species groups. (*C. leidy* is not included in this edition of the *Manual*.)

The initial system for classification of species of caulobacters (Poindexter, 1964; Poindexter and Lewis, 1966), continued in previous editions of this *Manual* (Poindexter, 1974, 1989), employed cell morphology as the principal subdividing criterion, in part because of the limited availability of other significant taxonomic criteria. More compelling, however, was the recognition that cell form under standard conditions is a complex and highly conserved feature of unicellular organisms; a feature such as cell pole contour is intimately associated with the reproductive mechanism, and mutations that affect cell shape can be lethal when expressed and often can be studied only in conditional mutants.

More than a decade of chemotaxonomic, ultrastructural, and genomic studies of the caulobacters has yielded taxonomically useful information that can now be added to morphological and

TABLE BXII.α.101. Differential morphology among genera that produce cylindrical prosthecae^a

Characteristic	Caulobacters ^b	<i>Prosthecoacter</i>	<i>Ancalomicrobium</i>	Budding hyphal bacteria ^c
<i>Reproduction:</i>				
Asymmetric binary fission	+			
Symmetric binary fission		+		
Budding from cell surface			+	
Budding from hyphal tip				+
Prosthecae per cell	1 (or 2 ^d)	1	3–4	1
Holdfast	+	+	–	+
Flagellum	1	0	0	1

^aFor symbols see standard definitions.^b*Caulobacter*, *Asticcacaulis*, *Maricaulis* *C. leidy*, and Group IV caulobacters.^c*Hyphomonas*, *Hyphomicrobium*, *Pedomicrobium*, and *Filomicrobium*.^dOne species of *Asticcacaulis* only.**TABLE BXII.α.102.** Differential characteristics among five species groups of caulobacters^a

Characteristic	<i>C. leidy</i>	<i>Caulobacter</i>	Group IV caulobacters	<i>Asticcacaulis</i>	<i>Maricaulis</i>
16S rRNA pattern ^b	I	III	IV	II	V
<i>Morphology:</i>					
Cells poles tapered	–	+	d	–	–
Cells distinctly curved	–	+	d	–	–
Stalk bands	–	+	+	+	–
S-layer	nd	+	–	nd	–
<i>Holdfast location on:</i>					
Stalk tip	+	+	+		+
Cell pole				+	
<i>Cellular pigments:</i> ^c					
None	+	+	+	+	+
Yellow	+	+	+	–	–
Gold, orange, red	–	–	+	–	–
Cellular lipid pattern ^d	nd	C	B	A	M
WGA-binding holdfast	–	+	+	+	+
<i>Vitamins known to stimulate growth of some isolates:</i>					
Riboflavin	–	+	–	–	–
Cyanocobalamin	–	+	–	–	–
Biotin	–	–	+	+	–
<i>Growth in media containing:</i>					
NaCl, 0% (w/v)	+	+	+	+	–
NaCl, 2% (w/v)	+	–	+	d	d
Sea salts, 1×	nd	–	d	–	+

^aSymbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; d, 11–89% of strains are positive; nd, not determined.^bSee Figs. BXII.α.111, BXII.α.114, and BXII.α.118.^c+, some isolates positive; –, no known isolates positive.^dSee generic descriptions of *Caulobacter* for lipid patterns B and C, of *Maricaulis* for lipid pattern M, and of *Asticcacaulis* for lipid pattern A.

nutritional traits in the continuing effort to recognize relationships among caulobacters and propose a phylogenetic classification.

As of 1 January 2001, 153 16S rDNA sequences of at least 1200 nucleotides in length, representing 71 caulobacter isolates and 39 isolates of hyphal budding bacteria, had been deposited with data banks. All 153 sequences, plus 24 sequences for other bacteria, were used to build a series of trees to guide this reconsideration of caulobacter classification. Neighbor-joining, maximum likelihood, and parsimony analyses repeatedly generated four major groups, which were further divisible into seven subgroups—five of caulobacters and two of hyphal budding bacteria. The tree shown in Fig. BXII.α.118 exemplifies the core topology of those trees. The tree displays the identity and location of 58 sequences, as follows: 1) all 37 sequences available for type strains of species and subspecies of caulobacters and hyphal budding bacteria that have been named or proposed; 2) 20 for strains for which sufficient phenotypic characterization is available to regard

them as dimorphic prosthecate bacteria and as representative of other unnamed isolates with similar descriptions and similar sequences; 3) a sequence for *Spirillum volutans*, a genus of *Alpha-proteobacteria* that occurs in caulobacter-laden waters. A neighbor-joining tree built using all 177 sequences is shown as groups of sequences in Fig. BXII.α.111. A DeSoete maximum-likelihood tree built using 98 sequences (trimmed here to 81: 57 nonredundant sequences for caulobacters and hyphal-budding bacteria, the 13 most similar sequences that could be found among nonprosthecate bacteria, 10 similar sequences for organisms of unknown phenotype, and the *S. volutans* sequence) is shown as groups of sequences in Fig. BXII.α.114. The relevant branches of this tree are expanded to display strain identity and sequence accession numbers for *Caulobacter* (Fig. BXII.α.112), Group IV caulobacters (Fig. BXII.α.113), *Asticcacaulis* (Fig. BXII.α.119), and *Maricaulis* (Fig. BXII.α.76 in the *Maricaulis* chapter), along with the positions of other similar sequences.

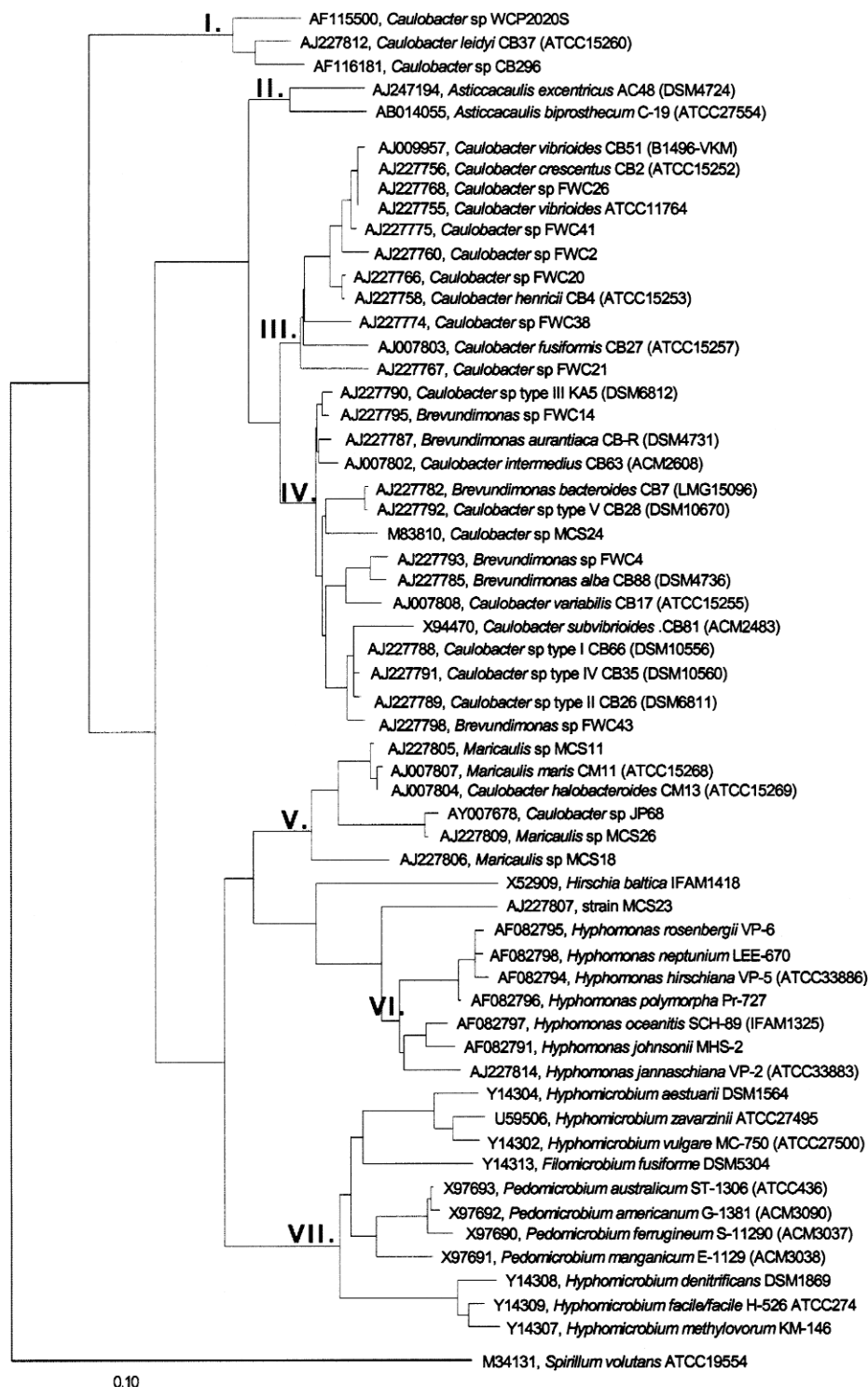


FIGURE BXII.α.118. Phylogenetic tree of 58 16S rDNA sequences of caulobacters (38), nonphototrophic hyphal-budding bacteria (19), and *Spirillum volutans* (1) built using maximum likelihood (PAUP 4.0) analysis. The correlations between 16S rDNA pattern and genus are as follows. I. *Sphingomonas* group (*C. leidy*), II. *Asticcacaulis*, III. *Caulobacter*, IV. Group IV caulobacters, V. *Maricaulis*, VI. *Hyphomonas*, VII. *Hyphomicrobium*. Bar indicates evolutionary distance.

The four major groups of caulobacters and hyphal budding bacteria reflect the natural distribution of these organisms: a pair of genera (*Maricaulis* and *Hyphomonas*) characteristically isolated from marine habitats; three species groups (*Caulobacter*, *Asticca-*

caulis, and the group IV caulobacters) encountered principally in freshwater environments; a diverse group (which includes *C. leidy*) isolated from soil, arthropod guts, desert soils, mine tailings, and fresh water that exhibit exceptional tolerance toward

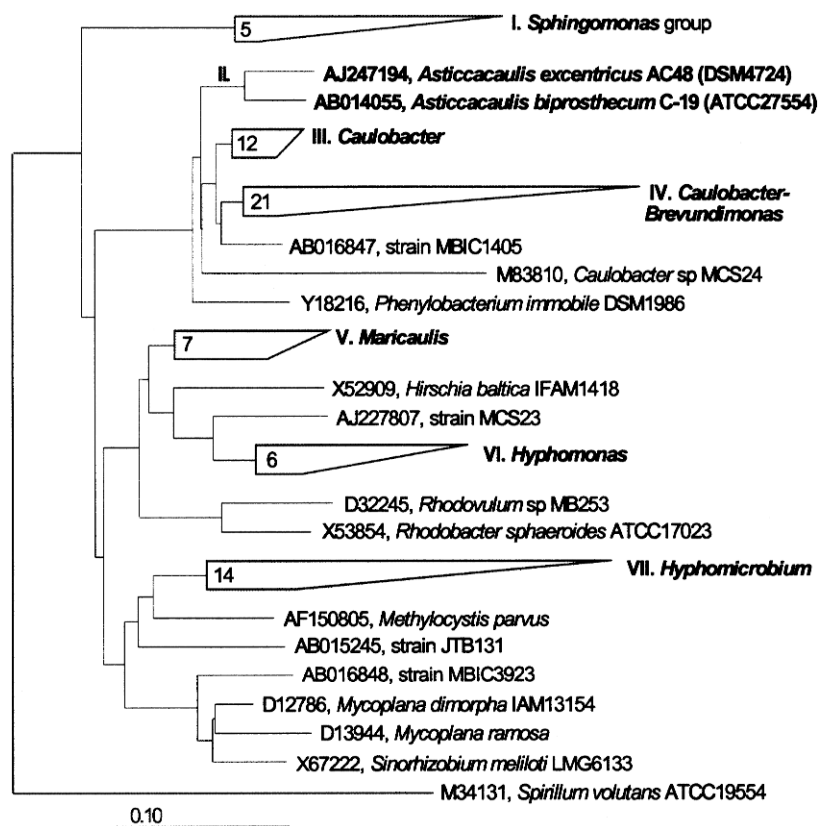


FIGURE BXII.α.119. Phylogenetic tree of 81 16S rDNA sequences, with group II. *Asticcacaulis* to display GenBank accession numbers and strain identities (see Fig. BXII.α.114 in the *Caulobacter* chapter). Also represented are the positions of sequences AB016610 and AF115499 (by AJ247194), and AF115501 and AJ247193 (by AB014055). Bar indicates evolutionary distance. Each enclosed numeral indicates the number of sequences grouped.

changes in environmental conditions; and oligocarbophilic, CO₂-dependent methylotrophs (*Hyphomicrobium*) that are widely distributed in soil, water, and sewage systems. Natural distribution and 16S rDNA sequence similarities correlate with differences in morphology, life cycle, certain nutritional and physiological properties, and details of ultrastructure and cell composition. Accordingly, they seem to constitute well-defined taxa, and the seven subgroups should be genera.

BLAST searches among GenBank sequences revealed different relationships outside the seven groups. Sequences like those of *C. leidy* are more similar to sequences of *Sphingomonas* species than to those of any of the other caulobacters, whereas the sequences of the two species of *Asticcacaulis* appear to be meaningfully similar only to sequences of *Caulobacter* and Group IV caulobacters. The sequence of one species of Gram-negative branching bacteria, *Mycoplana segnis*, is similar to some *Caulobacter* sequences. At least one strain of *M. segnis* contains cellular lipids similar to those found in *Caulobacter* isolates, but this organism is readily distinguished from caulobacters by its morphology, its lack of adhesiveness, its reproduction by multiple fragmentation, its peritrichous flagella, and much of its physiology. The sequences of other *Mycoplana* spp. group near other prosthecate bacteria: *M. bullata* near Group IV caulobacters and *M. dimorpha* and *M. ramosa* near *Hyphomicrobium*. Whether these sequence similarities imply an evolutionary relationship between branching and prostheca development cannot be assessed at present.

Some Group IV caulobacter sequences are noticeably similar to sequences of the two nonprosthecate species of *Brevundimonas*

(*B. diminuta* and *B. vesicularis*); *B. vesicularis*, in particular, also shares similarities of lipid composition with Group IV caulobacters. As suggested by Abraham et al. (1999), it is conceivable that the *Brevundimonas* species are of prosthecate ancestry, but are now "locked in the motile phase" (Abraham et al., 1999, p. 1070). However, general phenotypic similarity is low, and without the prosthecate species, *Brevundimonas* comfortably accommodates nonstalked, small rods that bear short-wavelength, polar flagella, divide symmetrically, and grow abundantly on rich media designed for the isolation and cultivation of clinically encountered microorganisms.

The greater similarity of sequences of the two marine genera to each other than to any of the nonmarine groups strongly suggests an independent evolutionary origin of the appendages in the marine organisms. The absence of bands from the stalks of marine caulobacters (Anast and Smit, 1988), as from all budding hyphae, supports the inference that the stalks of marine and freshwater caulobacters are less likely to be homologous structures than are the stalks and hyphae of *Maricaulis* and *Hyphomonas*, respectively. Unlike freshwater caulobacters, *Maricaulis* and *Hyphomonas* isolates share detectable 16S rDNA similarity with some purple photoheterotrophic bacteria (*Rhodospseudomonas*, *Rhodovulum*) and with members of the *Rhizobium* group. The sequences of nonmarine hyphal budding bacteria (the *Hyphomicrobium* group) are not significantly similar to any caulobacter sequences or to those of their marine morphological twins; they are more similar to sequences of nonhyphal methylotrophic bacteria (*Methylosinus* and *Methylocystis*). The 16S rDNA sequence

similarities between the two types of hyphal budding bacteria are of the same degree as similarities between each group and rhizobial sequences.

The 16S rDNA sequences imply that isolates previously assigned to *Caulobacter* represent three independent origins of the dimorphic life cycle characterized by asymmetric binary fission that produces a stalked cell and a monoflagellate swarmer cell—informally, “caulobacters.” To accommodate this implication, it is appropriate to redistribute former *Caulobacter* species and isolates among three groups. (1) *Maricaulis* (Abraham et al. 1999) has been proposed to accommodate the marine caulobacters. (2) A new genus will be proposed to accommodate *C. leidy* and other nonmarine caulobacters that appear to be more closely related to *Sphingomonas* than to any of the other caulobacters (Kurtz and Poindexter, unpublished). (3) The remaining caulobacter species and unassigned isolates appear to comprise a phylogenetically coherent group that also includes *Asticacaulis*. On the bases of relative similarities of 16S rDNA sequences and of phenotypic characteristics as shown in Table BXII.α.102, these non-marine caulobacters can be appropriately accommodated in three species groups: *Caulobacter* (rDNA group III) for classical vibrioid, colorless or lightly pigmented caulobacters as described (without isolates) by Henrici and Johnson (1935b), as well as one fusiform type; *Asticacaulis* (rDNA group II), which continues to accommodate colorless, rod-shaped caulobacters with stalks that are banded (as in the other two species groups), but do not bear the cell's holdfast; and a new genus that will be proposed (Kurtz and Poindexter, unpublished) to accommodate caulobacters of varied but rarely classically vibrioid cell shape that are often deeply pigmented and seem adapted to various levels of environmental salinity (rDNA Group IV).

This redistribution of caulobacters has been guided by investigations of 16S rDNA sequences and chemotaxonomic properties reported since the previous edition of this *Manual*, principally by the laboratories of W.R. Abraham, D.I. Nikitin, L.I. Sly, J. Smit, E. Stackebrandt, and D. Stahl (Anast and Smit, 1988; Merker and Smit, 1988; Stackebrandt et al., 1988a; Nikitin et al., 1990; MacRae and Smit, 1991; Stahl et al., 1992; Walker et al., 1992; Abraham et al., 1997, 1999; Sly et al., 1997, 1999). It is offered as a means of reducing heterogeneity within the genera used for the classification of these prosthecate bacteria and of resolving to some extent the question of the significance of cell morphology and life cycle as taxonomically useful traits. The accumulated work of several laboratories now implies a polyphyletic history of both caulobacter stalks and budding bacterial hyphae. Nevertheless, it is reasonable to propose that the detection of both nonmotile prosthecate and motile (mono-flagellated cells) in a pure population should be regarded as taxonomically helpful in assigning such an isolate to a taxon of phenotypically and genomically similar organisms.

Bacterial classification has become heavily dependent on genomic sequences, particularly of 16S rDNA, as one standard of taxonomic acceptability. Although genes descend and function within a genomic and cytoplasmic context, and one gene does not a genus make, sequences of 16S rRNA genes change very slowly and alert the systematist to spurious similarities of other characteristics that may well be instances of convergent evolution. They also aid in the recognition of phenotypic characteristics that are probably of phylogenetic import, and so help in interpreting the selective pressures that have influenced the course of evolution of a kind of organism. Without doubt, 16S rDNA sequences are sound and valuable guides to probable natural relationships, but they are neither all-powerful (Stackebrandt

and Goebel, 1994) nor—in practice—totally dependable. As this author delved into the sequences available for motile, prosthecate bacteria, several problems were discovered that have already led to confusion and occasional misinterpretation in caulobacter taxonomy. First, it was not at all uncommon to encounter sequences that had been determined for the same isolate (strain), but are currently listed and labeled in sequence banks as though that strain's sequence represents more than one species, even more than one genus! A second impediment to exploitation of this promising approach to microbial taxonomy was the difficulty of distinguishing between high similarity of phylogenetic significance and high similarity of techniques employed with the same strain obtained from different culture collections, labeled according to its shelf position in each collection rather than by the original label, which should accompany and identify the strain wherever it is used for any purpose. The current presence of unrecognizable strain redundancy encourages the discovery of “groups” created by unacknowledged label changes, not by natural selection.

The worst problem was the persistence in data banks of sequences that are mislabeled, probably because the cultures from which the DNA was extracted were either mislabeled or contaminated (see, e.g., comments in Sly et al. (1997), and in Abraham et al. (2001)). When such a sequence is detected, it would benefit every systematist who employs sequences if the label were corrected or the sequence withdrawn.

In preparation for this review of caulobacter classification, an effort was made to identify redundant sequences (those determined for the same strain and essentially identical) and sequences from probably mislabeled cultures. Of the 153 sequences deposited, 43 were regarded as recognizably redundant, and four were suspected to be mislabeled (M83797, recognized by Sly et al., 1997; M83796, recognized by Abraham et al., 1999; AJ007799 and AJ007800, recognized by Abraham et al. (2001)). While the overall approach and ultimate validity of 16S rDNA sequence analyses are undeniably valuable in systematics, there are pitfalls for the unwary attempting to use the expanding collection of data. It would be a great service to bacteriological (and probably all biological) systematics if someone regularly reviewed, and—with depositors' assent—removed or corrected the labels of sequences from misidentified cultures, cross-referenced sequences that purportedly display the same genomic region from the same strain, and otherwise edited, corrected, and updated taxonomic assignments with which sequences are labeled.

Lipid composition has become a taxonomically useful criterion in many bacterial groups, and the caulobacters are not an exception. Among these bacteria, there is a strong correlation between lipid composition and 16S rDNA sequence, providing two strong legs under the table on which to place three new (since the previous edition of this *Manual*) genera to accommodate isolates formerly or tentatively assigned to *Caulobacter*. Studies of caulobacterial lipids prior to the application of automated methods for lipid analysis (Chow and Schmidt, 1974; Carter and Schmidt, 1976; DeSiervo and Homola, 1980; DeSiervo, 1985) found evidence of two features of taxonomic importance: 1) fatty acids were not clearly distinctive among (then) *Caulobacter* species, and 2) marine caulobacters possessed significantly lower levels of phospholipids than did freshwater isolates. Differences in lipid composition were also detected between growing and stationary-phase cells.

More recently, technological developments have provided more detailed analysis of bacterial lipids, and the greatest detail regarding caulobacterial lipids has been reported by the labo-

ratory of W.R. Abraham (Abraham et al. 1997, 1999, 2001). Those studies reported fatty acid and polar lipid composition for isolates of four groups of caulobacters (*Caulobacter*, Group IV caulobacters, *Asticcacaulis*, and *Maricaulis*) and one of budding hyphal bacteria (*Hyphomonas*) in comparative studies that also included isolates of two nonprosthecae genera (*Brevundimonas* and *Mycoplana*). *C. leidy* was included, but seemed to be characterized mainly by the absence of identifiable polar lipids and the presence of C_{14:0} 2OH, an uncommon bacterial fatty acid that is, however, common among *Sphingomonas* spp. The two nonprosthecae types share some similarities with caulobacters in both lipid composition and 16S rDNA sequence. The major distinguishing features of the lipids detected are presented in both procedural and chemical detail in the reports from Abraham's group, which should be consulted. A similar composition was reported for one freshwater isolate by Batrakov et al. (1997). A brief summary of these reports is presented here.

Three patterns of lipid composition, designated "C", "B", and "M" by Abraham et al. (1997), appear to distinguish the three groups *Caulobacter*, Group IV caulobacters, and *Maricaulis*, respectively (see Table BXII.α.102), and a fourth pattern, designated "H", is found in *Hyphomonas*. The lipids of *Asticcacaulis* isolates appear to be somewhat more like those of *Caulobacter* than of the other genera. All groups, even *C. leidy*, share five fatty acids. The greatest differences, as anticipated by DeSiervo's studies, were detected between freshwater and marine caulobacters. The marine groups possess significant amounts of six fatty acids that are absent (five) or very low (the sixth) in freshwater organisms; the reverse was observed for three fatty acids—significant in freshwater, absent or scant in marine isolates.

Glucolipid patterns were also found to be distinctive. One type of phosphoglucolipid (PGL), 3-O-[6'-(sn-glycero-3'-phosphoryl)-α-D-glycopyranosyl]-sn-glycerols was the only such lipid found in the two *Asticcacaulis* and in 34 of 37 *Caulobacter* isolates tested. The exceptional *Caulobacter* isolates possess that PGL plus at least two others, as do 9 of the 13 Group IV caulobacter isolates and 1 of the 4 *Brevundimonas* strains. PGLs, at least one of which is present in every freshwater isolate, are scant or undetectable in marine isolates. Sulfonolipids were detected in many strains. All seven *Maricaulis* strains, 10 of 19 Group IV caulobacter strains, and two *Caulobacter* strains possess sulfoquinovosyl diacylglycerols (SQ), as do two of the four *Brevundimonas* strains and two marine isolates tentatively assigned to *Brevundimonas*. Sulfur-containing lipids were not found in *Asticcacaulis* isolates (Abraham et al., 2001). The *Hyphomonas* isolates lack SQ, but contain taurineamide diacylglycerides, found also in four *Maricaulis*, one *Caulobacter*, and one *Brevundimonas* strain. SQ are uncommon among nonphototrophic organisms, and it is remarkable and probably

taxonomically useful that they occur in both freshwater and marine caulobacters.

Seven kinds of bacteriophages have been isolated for caulobacters, two of which are not commonly encountered among noncaulobacters (Schmidt and Stanier, 1965; reviewed in Poindexter, 1981). The first of these is the most frequently reported type of caulophage; it is produced by strains of *Caulobacter*, Group IV caulobacters, and *Asticcacaulis*. The virions have large (50–65 × 170–260 nm), prolate, cylindrical heads and long (200–320 nm), flexible, noncontractile tails, and genomes of 2S DNA; they appear most like *Siphoviridae* among formal phage families. These phages exhibit wide host ranges among caulobacters; an individual phage may infect not only more than one species, but also more than one of the three freshwater species groups. The second, uncommon type consists of small icosahedral RNA phages (family *Leviridae*) produced by both *Caulobacter* and Group IV caulobacter isolates. Unlike the DNA-containing caulophages, each of the RNA phages is host species-specific, for *C. crescentus*, *C. fusiformis*, or *Brevundimonas bacteroides* (a Group IV caulobacter). Phages have not been reported to be produced by *Maricaulis* or *C. leidy*. None of the caulophages tested is lytic for non-caulobacters, and phages lytic for other genera are not lytic for caulobacters. Like most events in the life of caulobacters, the ability to adsorb and allow infection by some of the phages are traits expressed only intermittently during the cell cycle (Schmidt, 1966; Bendis and Shapiro, 1970; Lagenaur et al., 1977; Bender et al., 1989), usually during the swarmer stage. This is a further indication that asymmetric fission, with its intricate regulatory background and multiplicity of cell surface participants, continues to be the distinctive trait of caulobacters.

Four species of *Caulobacter* are recognized: *C. vibrioides*, *C. crescentus*, *C. henrici*, and *C. fusiformis*. Distinguishing and other traits are shown in Tables BXII.α.103 and BXII.α.104, respectively. The other nine species included in the previous edition of this *Manual* have been removed to other assignments: *C. bacteroides*, *C. henrici* subsp. *aurantiacus*, *C. intermedius*, *C. subvibrioides*, *C. subvibrioides* subsp. *albus*, and *C. variabilis* to *Brevundimonas* as six species; *C. halobacteroides* and *C. maris* to *Maricaulis*; and *C. glutinosus*, *C. kusnezovii*, and *C. leidy* to limbo.

ACKNOWLEDGMENTS

The author is deeply grateful to Dr. Thomas M. Schmidt of the Department of Microbiology and Molecular Genetics and Center for Microbial Ecology, Michigan State University, for his guidance in the analysis of 16S rDNA sequences and preparation of phylogenetic trees. His patience and generosity as a tutor are inexhaustible. The taxonomic conclusions, of course, are the responsibility of the author alone. The analysis and writing were supported in part by a Barnard College Faculty Grant during the author's sabbatical leave at Michigan State University in 2001.

List of species of the genus *Caulobacter*

1. ***Caulobacter vibrioides*** Henrici and Johnson 1935b, 84^{AL}. *vib.ri.oi' des.* M.L. n. *Vibrio* name of a genus; Gr. n. *eidus* form, shape; M.L. adj. *vibrioides* resembling a vibrio.

Vibrioid cells slender or nearly ovoid. Colonies colorless or pale yellow. Vitamin B₂ essential for growth, but additional unidentified growth factors available in peptone-yeast extract also required by most strains.

Strain CB51 was isolated from a freshwater pond in Berkeley, CA.

The mol% G + C of the DNA is: 64–65 (Bd, determined for two isolates).

Type strain: CB51, DSM 9893, VKM B-1496.

GenBank accession number (16S rRNA): AJ009957, AJ227754.

Additional Remarks: It is not clear whether any of the copies of strain CB51 currently available from culture collections is identical with the original isolate (Poindexter, 1964), which was not deposited with the ATCC. CB51 appears to have been or to have very early become overgrown by a strain of *C. crescentus*, resulting in identity of the 16S rDNA sequences that have been determined for CB51 and *C. crescentus* strain CB2, and DNA–DNA hybridization >90% (Moore et al., 1978). Response to riboflavin, the most easily tested distinction between the two species, has not been

TABLE BXII.α.103. Differential characteristics of the species of the genus *Caulobacter*^a

Characteristic	<i>C. vibrioides</i>	<i>C. crescentus</i>	<i>C. fusiformis</i>	<i>C. henricii</i>
Long axis distinctly curved	+	+	—	+
<i>Colonies:</i> ^b				
Colorless	+	+	—	—
Pale yellow	+	—	—	—
Bright yellow	—	—	—	+
Dark yellow	—	—	+	—
<i>Carbon sources generally used:</i>				
Carbohydrates	+	+	—	+
Amino acids	d	+	+	+
Other organic acids	d	+	—	d
Primary alcohols	—	+	—	—
<i>Organic growth factors required:</i>				
Riboflavin	+	—	—	—
Cyanocobalamin	—	—	—	+
Amino acids	—	—	—	d
Other, unidentified	+	—	+	—
Maximum specific rate of exponential growth >0.35 h ⁻¹	—	+	—	—

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative; and d, 11–89% of strains are positive.

^b+, some isolates positive; —, no known isolates positive.

TABLE BXII.α.104. Other characteristics of the species of the genus *Caulobacter*^a

Characteristic	<i>C. vibrioides</i>	<i>C. crescentus</i>	<i>C. fusiformis</i>	<i>C. henricii</i>
<i>Carbon-source utilization:</i> ^b				
Arabinose	+	d	—	d
Ribose	+	—	—	d
Xylose	+	+	—	+
Glucose	+	+	—	+
Galactose	+	+	—	+
Mannose	+	+	—	d
Fructose	+	—	—	d
Lactose	+	+	—	d
Maltose	+	+	—	+
Sucrose	+	+	—	+
Alanine	+	+	—	+
Aspartate	+	+	—	+
Glutamate	+	+	+	+
Proline	+	+	—	+
Tyrosine	d	+	—	d
Acetate	—	+	d	+
Butyrate	d	+	—	+
Pimelate	nd	+	—	+
Pyruvate	d	d	—	+
Malate	d	d	—	+
Fumarate	+	d	—	+
Succinate	+	—	—	+
Methanol	—	+	—	—
Ethanol	—	+	—	d
Propanol	—	+	—	—
Butanol	+	+	—	+
Pentanol	—	+	—	—
Starch hydrolysis	+	d	—	+
<i>Sensitivity to bacteriophages:</i> ^c				
Type I:				
Phages 1,3	—	d	—	—
Others	+	+	+	+
Type II	+	+	+	—
Type III	+	+	—	+
RNA phages	+	+	+	—

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative; d, 11–89% of strains are positive; and nd, not determined.

^bCarbon-source utilizations were determined with D-isomers of sugars, with L-isomers of amino acids, or (when single isomers were not available) with racemic mixtures.

^c2sDNA phage types: I, head prolate cylinder 50–65 × 170–260 nm, tail noncontractile 200–320 nm long; II, head elongated polyhedron 65–70 × 100–105 nm, tail noncontractile 260–300 nm long; III, head icosahedron 50–80 nm diameter, tail noncontractile 150–200 nm long. ssRNA phages: icosahedron 20–29 nm diameter, no tail.

reported for current copies of "CB51." As suggested by Sly et al. (1997), a search for original CB51 DNA should be made, or a substitute neotype strain matching the phenotype of *C. vibrioides* designated. Krasil'nikov and Belyaev (1973) reported 20 isolates of *C. vibrioides*. Babinchak and Gerencser (1976) reported 28 vibrioid isolates; however, characteristics other than morphology and phage sensitivities have not been described for their isolates. MacRae and Smit (1991) reported the isolation of 25 colorless vibrioid caulobacters from wastewater facilities; although they noted that many of the isolates were distinctly stimulated by supplementation of complex media with riboflavin, they did not indicate which of their isolates responded. Those isolates would be accommodated by *C. vibrioides*, whereas the riboflavin-indifferent isolates should be assigned to *C. crescentus*. Further colorless, vibrioid freshwater isolates were obtained from other sources and used by Merker and Smit (1988) and by Abraham et al. (1997, 1999), but these were not significantly different from each other or the wastewater isolates in lipid composition. Different strains in the collection did, however, bind different plant lectins, a trait that might eventually prove to be an additional aid in distinguishing between the two colorless vibrioid species, *C. vibrioides* and *C. crescentus*.

2. **Caulobacter crescentus** Poindexter 1964, 288^{AL}

cres' cen. tus. L. adj. *crescentus* of the moon in its first quarter, crescent.

Vibrioid cells slender (see Fig. BXII.α.115). Colonies colorless, with centers becoming tan or dark pink upon aging. Lack of growth factor requirements is unique among *Caulobacter* species, as is the ability to utilize primary alcohols as sole sources of carbon and energy in defined media; other species may utilize butanol, but not methanol, ethanol, propanol or pentanol, as do *C. crescentus* isolates. Some of the wastewater caulobacters isolated by MacRae and Smit (1991) may be *C. crescentus*, but this species cannot be distinguished from *C. vibrioides* by the traits (lipid composition, 16S rDNA sequences, lectin binding) so far reported for these isolates.

Strain CB2 was isolated from tap water in Berkeley, CA.

The mol% G + C of the DNA is: 62–67 (Bd, determined for one isolate; *T_m*, determined for three isolates; paper electrophoresis of hydrolysate, determined for one isolate; complete sequence for one isolate).

Type strain: CB2, ATCC 15252.

GenBank accession number (16S rRNA): AJ227756.

3. **Caulobacter fusiformis** Poindexter 1964, 289^{AL}

fus. i. form' is. L. n. *fusus* spindle; L. n. *forma* shape, form; M.L. adj. *fusiformis* spindle-shaped.

Fusiform cells slender (see Fig. BXII.α.116). Colonies bright yellow. Sugars not utilized as carbon sources. Organic growth factor requirements not satisfied by mixtures of B vitamins, amino acids, and purine and pyrimidine bases.

Strain CB27 was isolated from a freshwater pond in Berkeley, CA.

The mol% G + C of the DNA is: not determined.

Type strain: CB27, ACM 5108, ATCC 15257, DSM 4728.

GenBank accession number (16S rRNA): AJ227759, AJ007803, AB008533.

Additional Remarks: Krasil'nikov and Belyaev's (1973) strain 25 (VKM v-1183) was assigned by them to this species. However, based on its morphology, pigmentation, and ability to utilize a variety of sugars, it is assignable to Group IV caulobacters. Another fusiform isolate, strain 1 (VKM v-1189), designated "*Caulobacter rossii*" sp. nov. by Krasil'nikov and Belyaev (1973), is not assignable to *C. fusiformis*; because only one such isolate is known and its physiological characteristics are predominantly inabilities and appear variable, "*C. rossii*" is not included in this list. Babinchak and Gerencser (1976) reported five fusiform isolates; however, characteristics other than morphology and phage sensitivities have not been described for their isolates. Yellow fusiform isolates were not obtained from wastewater by MacRae and Smit (1991).

4. **Caulobacter henricii** Poindexter 1964, 288^{AL}

hen. ric' i. i. M.L. gen. n. *henricii* of Henrici; named for A.T. Henrici, who observed stalked bacteria on slides that had been submerged in freshwater.

Vibrioid cells slender. Colonies bright yellow and may be somewhat translucent. Vitamin B₁₂ typically required as growth factor in peptone–yeast extract media.

Strain CB4 was isolated from a freshwater pond in Berkeley, CA.

The mol% G + C of the DNA is: 62–65 (Bd, determined for two isolates).

Type strain: CB4, ATCC 15253, DSM4730, ACM5105.

GenBank accession number (16S rRNA): AJ227758, AJ007805, AB008532.

Additional Remarks: Krasil'nikov and Belyaev's (1973) strain 44 (VKM v-1190), designated "*Caulobacter rutilus*" sp. nov. is not distinguishable from *C. henricii*. As described here, *C. henricii* accommodates all known *Caulobacter* isolates of yellow-pigmented, riboflavin-indifferent vibrioid types. Only two wastewater isolates (FWC20 and FWC23) reported by MacRae and Smit (1991) were yellow and vibrioid; the 16S rDNA sequence of one (FWC20) has been determined and found to be more similar (99.8%) to the type strain of *C. henricii* than to any other caulobacter sequence.

Genus II. Asticcacaulis Poindexter 1964, 282^{AL}

JEANNE S. POINDEXTER

A' stic. ca. cau' lis. Gr. *alpha* privative without; Anglo-Saxon n. *sticca* stick; L. n. *caulis* stalk; L. masc. n. *Asticcacaulis* stalk that does not stick.

Cells single, unbranched, **rod-shaped**, poles blunt or gently rounded; 0.5–0.7 × 1–3 μm during growth in most media. Some cells in any growing population have one subpolar or one or two lateral **prosthecae**. Each prostheca includes outer membrane, peptidoglycan, cell membrane, and a core sometimes observed to be occupied in part by membranes, but other cytoplasmic

components cannot be discerned through most of its length. Beyond the cell–prostheca juncture, prostheca diameter is constant, 0.10–0.15 μm; each prostheca possesses at least one **stalk band** as a substructure after the cell has matured and completed at least one cell cycle. Other cells in the same population bear a **single, subpolar flagellum**. Each type of cell bears a small mass

of adhesive material, the **holdfast**, at one pole; the holdfast site is not coincident with the site of the flagellum or of the prostheca(e). Binary fission occurs by **septation**, typically resulting in the production of a longer, prosthecae cell and a shorter, flagellated cell. Fission may occur in cells lacking prosthecae. In both instances, **cell division is unequal**.

Gram negative. Strictly **respiratory** and **aerobic** but may be somewhat O₂ sensitive; O₂ serves as the only terminal electron acceptor for growth, although nitrate may be reduced to nitrite. Colonies circular, convex, glistening, with smooth margins, butyrous in texture, and colorless. In standing liquid cultures, cells accumulate as a **surface film** or heavier pellicle and develop as a ring of growth on the vessel wall at or just below the air-liquid interface. Growth in agitated liquid cultures is evenly dispersed.

Chemoorganotrophic and **oligotrophic**; grow readily in media such as peptone-yeast extract containing 0.05–0.3% (w/v) organic solutes, but not in standard nutrient broth with 0.8% (w/v) organic solutes. During growth, may produce acid from sugars but do not produce gas. Optimal temperature for growth: 25–30°C; tolerated range for growth: 15–35°C. Optimal pH near neutrality; pH 6–9 tolerated. Typically do not grow in media containing 2% (w/v) NaCl. Maximum specific rates of exponential growth: 0.23–0.57 h⁻¹. All isolates require biotin as the only organic micronutrient. Glucose, fructose, maltose, or lactose may be utilized as the sole carbon source.

Sequences of 16S rDNA are consistent with placement of *Asticcacaulis* among the *Alphaproteobacteria*. The positions of the six sequences grouped as *Asticcacaulis* in Fig. BXII.α.111 in the chapter describing the genus *Caulobacter* are represented in Fig. BXII.α.119 (in the *Caulobacter* chapter) by the two nonredundant sequences grouped in Fig. BXII.α.114 in *Caulobacter*; their strain identities and GenBank accession numbers are displayed. These sequences were determined for the type strain of each species. (See “Taxonomic Comments” in the chapter describing the genus *Caulobacter* for a description of this tree.)

Sources: isolated from tap water and natural bodies of fresh water. Not encountered as clinical isolates, and not known to be pathogenic for plants or animals. Rarely encountered; only four isolates of *Asticcacaulis excentricus* are known, and only one of *Asticcacaulis biprosthecum* has been described.

The mol% G + C of the DNA is: 55–61.

Type species: *Asticcacaulis excentricus* Poindexter 1964, 292.

FURTHER DESCRIPTIVE INFORMATION

Morphology and fine structure The prosthecae of all isolates are banded (see Figs. BXII.α.120B and BXII.α.121C). Each band is a set of concentric rings that are more electron-opaque and structurally more rigid than is the remainder of the prostheca (Schmidt and Swafford, 1975). Earlier evidence that one band is added to each growing prostheca during each cell cycle of *Caulobacter crescentus* (Staley and Jordan, 1973) has been confirmed for both that species and for *Asticcacaulis biprosthecum* (Poindexter and Staley, 1996). However, the function of the bands is unknown. They occur in the prosthecae of only three species groups: *Caulobacter*, *Asticcacaulis*, and “other caulobacters.”

The pattern of prostheca development in *Asticcacaulis* species as it relates to the cell cycle appears to vary among isolates and, in a given isolate, with composition of the growth medium (Pate et al., 1973; Larson and Pate, 1975). In some cultures, development occurs only toward the end of exponential growth; in others, practically all dividing cells are prosthecae in all growth

phases of the culture. As far as is known, only one progeny cell of each reproductive event is flagellated, whether or not the other progeny cell bears a prostheca. Prosthecae that arise laterally are usually inherited by the nonmotile progeny cell, but may also be inherited by the motile cell. However, the capacity for development is not lost by a clone arising from a nonmotile, nonprosthecae cell; clones without prosthecae have not been encountered as spontaneous variants. As with other caulobacters, prostheca length is greatly increased in dilute media; prostheca length is also promoted in defined media, especially when phosphate concentration is growth limiting (Schmidt and Stanier, 1966), but also when the available carbon source is only slowly utilized (Larson and Pate, 1975).

Cellular composition The thin peptidoglycan layer of the *Asticcacaulis* cell body continues without interruption into the prostheca. Peptidoglycan of both known species is similar with respect to the glycan component, in which muramic acid is present in a 50% excess over glucosamine (Poindexter and Hagenzieker, 1982). The species, however, differ in peptide composition. *A. biprosthecum* contains a significantly higher proportion of glutamic acid than is found in *A. excentricus* and other Gram-negative bacteria. Prostheca peptidoglycan has not been separately assayed in either species.

The principal respiratory quinone in *Asticcacaulis* cells is ubiquinone-10, and the principal phospholipid is phosphatidylglycerol. The most abundant fatty acid species are C_{18:1} and C_{16:0}. Both isolates tested (one of each species) also contain lower, but significant amounts of C_{18:1 ω7c} 11CH₃ and of species of C_{17:1} and C_{15:0}. As in *Caulobacter*, the principal hydroxy fatty acid detected by Abraham et al. (2001) was C_{12:1 3OH}, although a study of the lipid composition of an isolate described as “*A. biprosthecum*-like” (Sittig and Schlesner, 1993) reported detection of C_{12:0 3OH}, as well. Neither isolate studied by Abraham et al. (2001) contained the PGLs found in *Caulobacter* and “other caulobacters.”

Enzymes Intermediary metabolism has been examined in only a preliminary fashion. The Entner–Doudoroff pathway for carbohydrate dissimilation is present. Enzymes for catabolism of some carbohydrates are induced by exposure to substrate, but induced levels are only 1.5–10-fold higher than uninduced levels.

Genome structure DNA–DNA hybridization values were reported by Moore et al. (1978) to be in the range of 33–88% among isolates of *A. excentricus*, but to be undetectable between the type strains of the two species in this genus. Ribosomal rDNA sequences have been determined for both species of *Asticcacaulis*, which appear to comprise one of five coherent groups among caulobacterial sequences. The minimum similarity between the sequences that represent the two isolates grouped within “II. *Asticcacaulis*” in Fig. BXII.α.111 in the *Caulobacter* chapter is 95.9%. All known sequences for 16S rDNA are less similar; the highest similarities outside the genus are with sequences for organisms identified as *Caulobacter henricii* (93.7%) or *Brevundimonas aurantiaca* (formerly *C. henricii* subsp. *aurantiacus* (94.7%)).

Three morphological types of bacteriophages that are lytic for *Asticcacaulis* species have been isolated from fresh water and sewage (Pate et al., 1979; reviewed in Poindexter, 1981). All are two-stranded DNA phages with long, flexible, noncontractile tails. Heads may be icosahedral, prolate cylindrical, or elongated polyhedral. Of more than 40 phage isolates known from *Asticcacaulis*, only two (of the prolate cylindrical type) are lytic for a few *Caulobacter* and “other caulobacter” isolates; all others are lytic only

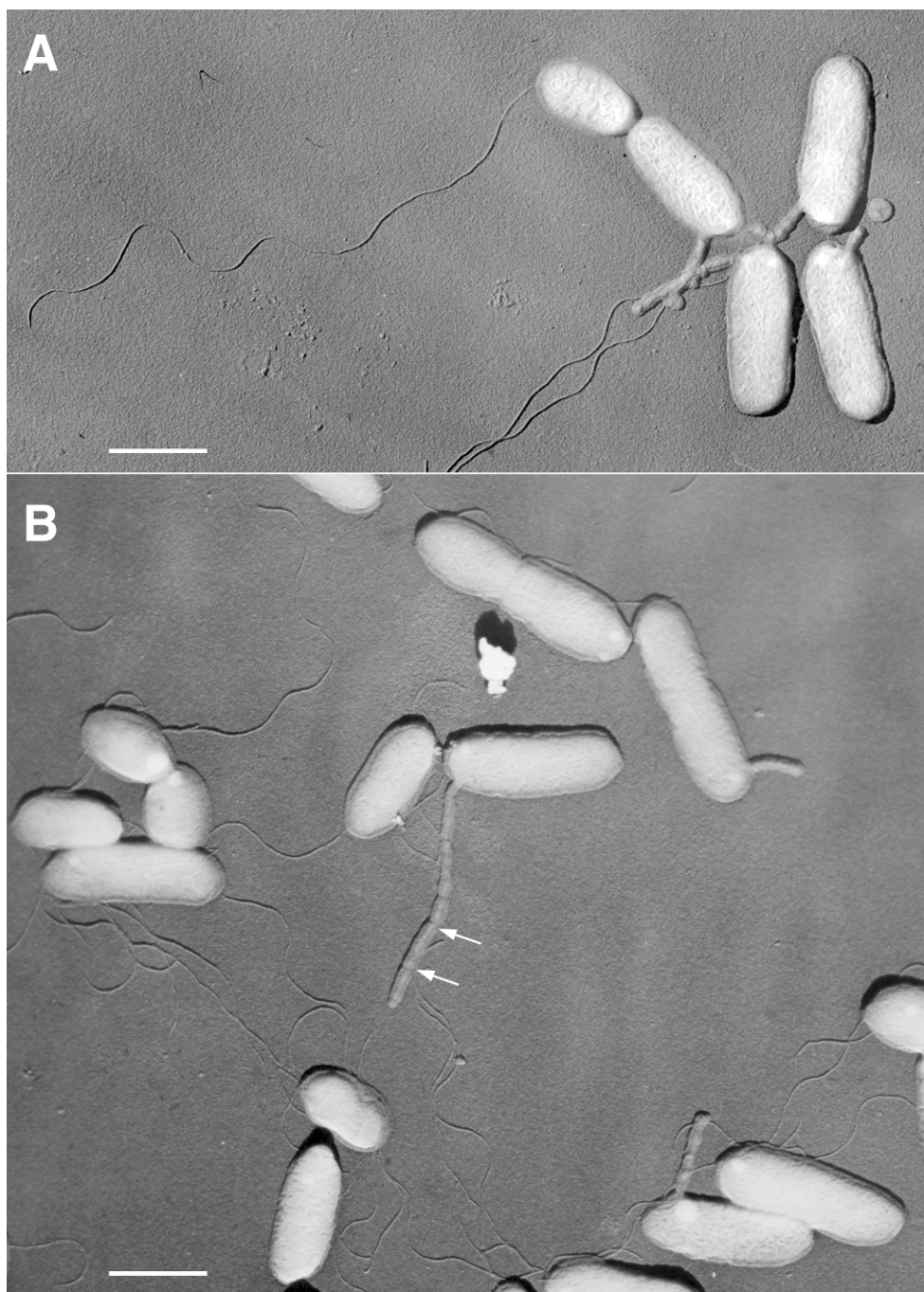


FIGURE BXII.α.120. *A. excentricus* strain S-3 grown in peptone–yeast extract medium. Electron microscopy, shadowed specimen, negative image. Arrows in (B) indicate stalk bands. Bars = 1 μ m.

for *Asticcacaulis* isolates. No phages that are lytic for any other genus (including other caulobacters) are also lytic for *Asticcacaulis* isolates. Generally, this genus appears to be isolated from other genera with respect to phage propagation. Genetic studies have not been done for this genus, but *A. excentricus* has been found to be capable of accepting and expressing plasmid-borne genes, including genes for insect larvicide production (Liu et al., 1996).

See “Taxonomic Comments” below and in the chapter describing the genus *Caulobacter* for further discussion of 16S rDNA sequences and other bacteriophages.

ENRICHMENT AND ISOLATION PROCEDURES

Asticcacaulis is not frequently observed in natural samples and is rarely isolated. Isolation has been achieved by the same procedure as described for *Caulobacter* (*q.v.*). A sample of clean water is enriched with at most 0.01% (w/v) peptone and allowed to stand at room temperature. Within a few days, a sample of the surface film, taken with a bacteriological loop or a cover slip, is examined by phase-contrast microscopy for the presence of prosthecate cells or rosettes of rod-shaped cells. When such cells account for 10–50% of the population, a sample (again, taken with a loop, not a pipette) is streaked on a dilute (0.05%; max-

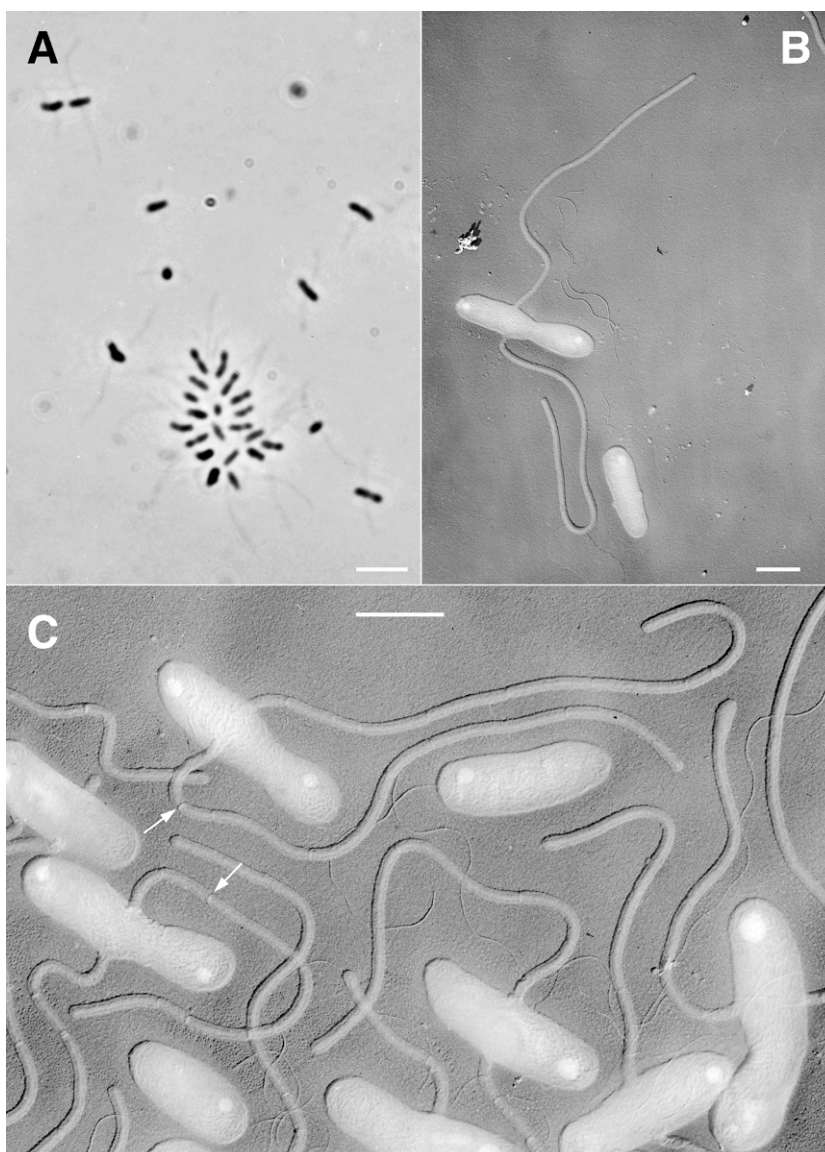


FIGURE BXII.α.121. *A. biprosthecum* strain C-19 grown in peptone–yeast extract medium. *A*, phase-contrast microscopy, wet mount. *B–C*, electron microscopy, shadowed specimen, negative image. Arrows in (*C*) indicate stalk bands. Bars = 5 μ m (*A*) and 1 μ m (*B–C*).

imum 0.1%, w/v) peptone or peptone–yeast extract medium prepared with 1.0 or 1.5% agar. By the third or fourth day of incubation of the plates, small, hyaline or crystalline, noniridescent colonies of prosthecate bacteria begin to appear. These colonies, or patches of growth inoculated from them, are then screened by phase-contrast microscopy. Clones of *Asticcacaulis* are often difficult to recognize during the microscopic screening. However, any clone that includes frequent rosettes should be purified, cultivated in dilute (e.g., 0.1% peptone, 0.05% yeast extract) broth, and examined by electron microscopy for the presence of prosthecate and flagellated cells in whole-cell mounts, such as shadowed or negatively stained specimens.

A. biprosthecum has been isolated only once, by a modification of this procedure in which a loopful of the enrichment culture was streaked on a dilute medium containing 0.02% beef extract, 0.05% tryptone, 0.05% yeast extract, and 0.02% sodium acetate, prepared with 1.5% agar. After several days of incubation at 30°C,

very small colonies were transferred as deep cultures to the same medium prepared with 0.4% agar. *A. biprosthecum* was detected by microscopic examination of the submerged colonies (Pate et al., 1973).

MAINTENANCE PROCEDURES

Once isolated, *Asticcacaulis* species can be maintained as vegetative, frozen, or lyophilized populations. Vegetative stocks should be maintained on 1% agar slants of dilute (0.05–0.3% organic material) complex medium, transferred every 8 or 9 weeks, incubated 2 or 3 days at 20–25°C, and then refrigerated. Cells grown in dilute complex medium can be stored in small volumes frozen at –70°C without cryoprotectant. Such frozen cultures can be thawed at room temperature and transferred to growth medium to resume vegetative growth. Lyophilization is a dependable means for maintenance, either in milk solids or on strips of filter paper.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

This genus is distinguished by the number, positions, and substructures of its appendages, particularly the prosthecae. The appendages are best examined by electron microscopy. Shadowed specimens provide the clearest image, but negatively stained specimens need not be washed and so preserve a higher proportion of attached flagella. The holdfast is best detected by its adhesive function, although it, too, is discernible by electron microscopy.

DIFFERENTIATION OF THE GENUS *ASTICCACAULIS* FROM OTHER GENERA

Four groups of bacteria other than caulobacters produce long cellular appendages of the envelope that are of constant diameter; these bacteria also tend to occur in the same habitats as caulobacters. They are distinguishable from caulobacters and from each other by the morphology of their reproductive stages. The five groups are distinguished in Table BXII.α.101 in the *Caulobacter* chapter, and the five genera of caulobacters are distinguished in Table BXII.α.102 in the *Caulobacter* chapter. Relationships to nonprosthecae bacteria implied by 16S rDNA analysis are illustrated and discussed further in "Taxonomic Comments" in the chapter describing the genus *Caulobacter*.

TAXONOMIC COMMENTS

The genus *Asticcacaulis* was created (Poindexter, 1964) to accommodate isolates originally regarded as *Caulobacter* species, but whose prosthecae were not adhesive and consequently not equivalent to *Caulobacter* stalks as adhesive organelles. Subsequent investigations of a greater variety of prosthecae bacteria have revealed that only caulobacterial appendages lack cytoplasm along most or all of their length and that stalk bands occur only in freshwater caulobacters. The unique structure of the freshwater caulobacter appendages suggests that they are homologous. Although the mol% G + C of *Asticcacaulis* DNA is lower than that known for other freshwater caulobacters, and only two of more than 100 caulophage isolates are lytic for both *Asticcacaulis* and other freshwater caulobacters, 16S rDNA sequence analysis places *Asticcacaulis* well within the caulobacter group (*Caulobacter*,

Asticcacaulis, and "other caulobacters"). Its closest nonprosthecae relative is *Brevundimonas*, whose sequences are more similar to "other caulobacter" sequences than to those of *Asticcacaulis*.

Two species are recognized and are distinguished by the number and position of the prosthecae (Table BXII.α.105); other characteristics are listed in Table BXII.α.106. Individual strains of the icosahedral-head DNA phages are lytic for both species, but cellular genomic DNA-DNA similarity has not been detected (Moore et al., 1978).

TABLE BXII.α.106. Other characteristics of the species of the genus *Asticcacaulis*^a

Characteristic	<i>A. excentricus</i>	<i>A. biprosthecum</i>
<i>Carbon-source utilization</i> : ^b		
Arabinose	d	—
Ribose	—	nd
Xylose	+	+
Glucose	+	+
Galactose	+	+
Mannose	+	—
Fructose	+	+
Lactose	+	+
Maltose	+	+
Sucrose	+	—
Alanine	+	+
Aspartate	+	+
Glutamate	+	+
Proline	+	+
Tyrosine	d	—
Acetate	+	—
Butyrate	—	nd
Pimelate	—	nd
Pyruvate	+	+
Malate	+	—
Fumarate	+	—
Succinate	+	—
Methanol	d	—
Ethanol	+	+
Propanol	d	—
Butanol	d	—
Pentanol	d	—
Starch hydrolysis	+	+
<i>Sensitivity to bacteriophages</i> : ^c		
Type I:		
Phages 1, 3	—	—
Others	+	+
Type II	—	+
Type III	+	+
RNA phages	—	—

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative; d, 11–89% of strains are positive; nd, not determined.

^bCarbon-source utilizations were determined with D-isomers of sugars, with L-isomers of amino acids, or (when single isomers were not available) with racemic mixtures.

^c2sDNA phage types: I, head prolate cylinder 50–65 × 170–260 nm, tail noncontractile 200–320 nm long; II, head elongated polyhedron 65–70 × 100–105 nm, tail noncontractile 260–300 nm long; III, head icosahedron 50–80 nm diameter, tail noncontractile 150–200 nm long. ssRNA phages: icosahedron 20–29 nm diameter, no tail.

TABLE BXII.α.105. Differential characteristics of the species of the genus *Asticcacaulis*^a

Characteristic	<i>A. excentricus</i>	<i>A. biprosthecum</i>
<i>Typical number of prosthecae per cell</i> :		
One	+	—
Two	—	+
<i>Position of prostheca</i> :		
Subpolar	+	—
Lateral	—	+

^aSymbols: +, 90% or more of strains are positive; and —, 90% or more of strains are negative.

List of species of the genus *Asticcacaulis*

1. *Asticcacaulis excentricus* Poindexter 1964, 292^{AL}
ex.cen'tri.cus. L. pref. *ex* out, beyond; Gr. n. *centron* center of circle; M.L. adj. *excentricus* out from the center.

Rod-shaped cells thick (see Fig. BXII.α.120). A single prostheca arises from a subpolar site previously occupied by a single flagellum. Colonies colorless. Biotin is the only growth factor required in glucose-ammonium salts me-

dium. Sugars are the preferred carbon sources; all isolates can also use ethanol, but use of other primary alcohols is variable among isolates.

Strain AC48 was isolated from a freshwater pond in Berkeley, CA.

The mol% G + C of the DNA is: 55–60 (Bd, *T_m*).

Type strain: AC48, ACM 1263, ATCC 15261, DSM 4724.

GenBank accession number (16S rRNA): AJ247194.

2. ***Asticcacaulis biprosthecum*** Pate, Porter and Jordan 1973, 582^{AL}.

bi.pros.thec'um. L. pref. *bi, bis* twice; Gr. fem. n. *prosthece* appendage; M.L. adj. *biprosthecum* twice-appendage(d).

Rod-shaped cells thick (see Fig. BXII.α.121). One or, more often, two prosthecae arise near the equator of the cell at roughly diametrically opposite positions from each other; neither site is coincidental with the site occupied by the single, subpolar flagellum. Colonies colorless. Biotin is the only growth factor required in glucose–ammonium salts medium, but growth is markedly stimulated by mixtures of amino acids; supplementation with single amino acids can be inhibitory. Somewhat sensitive to dissolved O₂, growing faster and more efficiently (as measured by yield) when

allowed to reduce medium prior to aeration. Pili as well as holdfast material participate in adhesion.

Strain C-19 was isolated from a freshwater pond in Madison, WI.

The mol% G + C of the DNA is: 61 (*T_m*).

Type strain: C-19, ACM 2498, ATCC 27554, DSM 4723, MBIC 3411.

GenBank accession number (16S rRNA): AJ247193.

Additional Remarks: There is a single known isolate of this species; strain C-19 was the original strain designation, but this isolate also appears in the literature as AC-2. One "*A. biprosthecum*-like" isolate has been reported (Sittig and Schlesner, 1993). (Note: The species epithet was misspelled "biprosthecium" in the 1989 Approved List; the spelling originally proposed by Pate et al., 1973, should be used.)

Genus III. *Brevundimonas* Segers, Vancanneyt, Pot, Torck, Hoste, Dewettinck, Falsen, Kersters and De Vos 1994, 507^{VP} emend. Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennisar, Smit and Tesar 1999, 1070

MARC VANCANNEYT, PAUL SEGERS, WOLF-RAINER ABRAHAM AND PAUL DE VOS

Brev.un.d'i'mo.nas. L. adj. *brevis* short; L. fem. n. *unda* wave; Gr. *monas* a unit, monad; M.L. fem. n. *Brevundimonas* bacteria with short wavelength flagella.

Gram-negative, rod-shaped, subvibrioid or vibrioid cells, 0.4–0.5 × 1–2 µm. **Cells of some species can form prosthecae (stalks).** These species are characterized by an asymmetric cell division whereby fission results in a prosthecate (nonmotile) and a flagellated (motile) cell. Motility by means of single polar flagella. Nonsporeforming. A yellow or orange carotenoid pigment may be formed.

Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Catalase positive. Nitrate is rarely reduced.

Chemoorganotrophic and oligotrophic. Good growth occurs on most common media at a pH range of 6.0–8.0 and at 25–30°C. **Growth factors are required by all strains.** Growth occurs without NaCl, but is optimal at 0.5–2% NaCl (w/v). All species can utilize pyruvate, and most isolates are also positive for growth on the organic acids acetate, butyrate, fumarate, and succinate and on the amino acids glutamate and proline. Glucose, galactose, maltose, and starch can be utilized by all species except the type species, which has a restricted nutritional spectrum. Growth on primary alcohols is usually negative. No or weak acid production from sugars.

Dominant fatty acids are C_{12:0} 3OH, C_{14:0}, C_{15:0}, C_{16:0}, C_{16:1}, C_{17:0}, C_{17:1} ω6c, C_{17:1} ω8c, and C_{18:1}. Polar lipids are α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol, 1,2-di-O-acyl-3-O-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl] glycerol, 6-phosphatidyl-α-D-glucopyranosyl diacylglycerol (main mass numbers 1413 and 1439 Da), and phosphatidylglycerol. Most strains contain sulfoquinovosyl diacylglycerol. Ubiquinone Q-10 and the polyamines spermidine and homospermidine are present in the species examined. Cells accumulate poly-β-hydroxybutyrate as a reserve material but are, so far as known, not able to hydrolyze this polymer. The genus belongs to the class *Alphaproteobacteria*. Strains are isolated from water, soil, and clinical specimens.

The mol% G + C of the DNA is: 62–68.

Type species: ***Brevundimonas diminuta*** (Leifson and Hugh 1954b) Segers, Vancanneyt, Pot, Torck, Hoste, Dewettinck, Fal-

sen, Kersters and De Vos 1994, 507 (*Pseudomonas diminuta* Leifson and Hugh 1954b, 68.)

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic treatment Within the class *Alphaproteobacteria*, *Brevundimonas* belongs to the family *Caulobacteraceae*, which also includes the genera *Asticcacaulis*, *Caulobacter*, and *Phenylobacterium*. *Brevundimonas* is most closely linked to *Caulobacter*, with 16S rDNA sequence similarities of 95–96% (Fig. BXII.α.122; Abraham et al., 1999). Lower similarities of 93–94% and 92–93% are obtained with *Asticcacaulis* and *Phenylobacterium*, respectively. Within *Brevundimonas*, the interspecies 16S rDNA sequence similarities are higher than 96%. Remarkably, the misclassified species, "*Mycoplana bullata*", groups among the *Brevundimonas* species, with similarities above 97% (see below). No phylogenetic subgroups are recognized that reflect the distinct morphological characteristics of the species (see below; Fig. BXII.α.122; Abraham et al., 1999).

Cell morphology Cell morphology may be a primary criterion for distinguishing between the dimorphic prosthecate and nonprosthecate *Brevundimonas* species. Reproduction of six prosthecate species, *Brevundimonas alba*, *Brevundimonas aurantiaca*, *Brevundimonas bacterioides*, *Brevundimonas intermedia*, *Brevundimonas subvibrioides*, and *Brevundimonas variabilis*, results in the separation of two cells that are morphologically and behaviorally different from each other. Cells divide by binary transverse fission (which is constrictive without formation of a septum), in which the younger pole bears a single flagellum, and the older pole bears a prostheca derived from the cell envelope. The stalk includes membranes and peptidoglycan, but not cytoplasmic components. The stalk-bearing progeny cell grows and eventually repeats the asymmetric cell division. The flagellum-bearing progeny cell, after a period of motility, releases the flagellum and develops its stalk at the previously flagellated site as it grows and proceeds to its asymmetric cell division (Poindexter, 1989). At the base of the flagellum and at the outer tip of the stalk is a

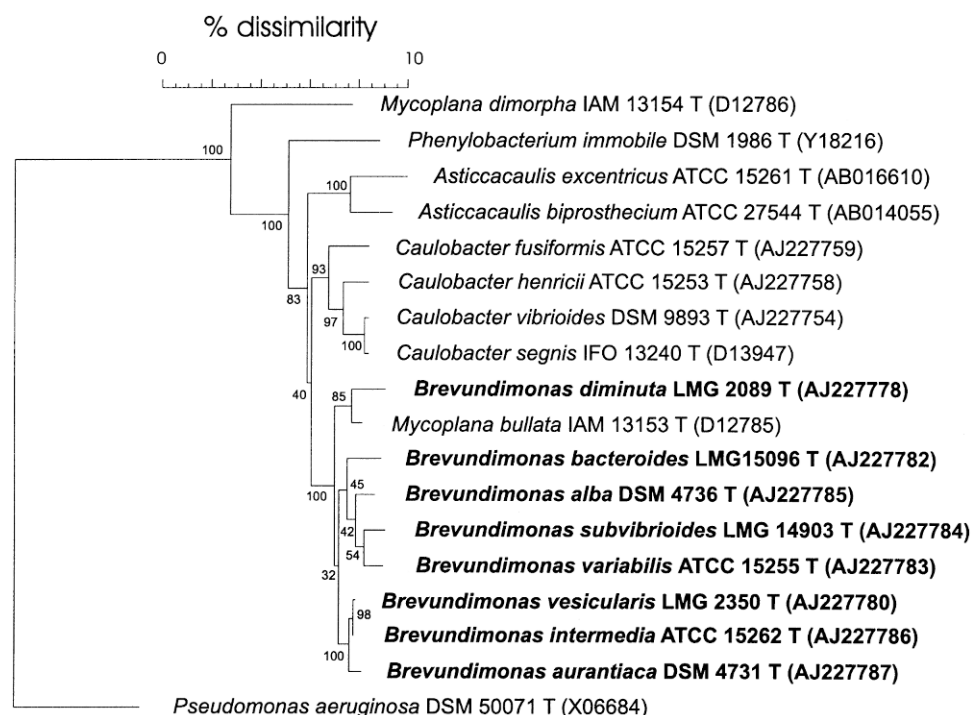


FIGURE BXII.α.122. Phylogenetic tree based on a comparison of 16S rDNA sequences of strains belonging to the genera *Brevundimonas* and related taxa (accession numbers are given in brackets). T, indicates type strain.

small mass of adhesive material, the holdfast, which confers adhesiveness on each of the progeny (Poindexter, 1989). Stalk position is species-specific or, for one species, may vary among strains, but the stalk length varies greatly among isolates (0.11–0.18 μm ; Poindexter, 1989). The cell is rod-shaped, vibrioid, or subvibrioid, depending on the species, but cell shape may be influenced by medium composition (e.g., phosphate concentration) or culture age (Poindexter, 1989).

Strains of the nonprosthecate species, *Brevundimonas diminuta* and *Brevundimonas vesicularis*, are short rods with an unusual short-wavelength flagellar morphology (average wavelength, 0.62 μm). The flagellum originates for most cells from the periphery of the pole rather than from the center of the pole (Leifson and Hugh, 1954b; Clark et al., 1984).

Cultural characteristics Colonies are usually circular, convex, and glistening with smooth margins. Colony diameter varies with strain and medium, but usually reaches 3–5 mm after several days of incubation. Colonies are colorless or may have a pink, yellow, orange, or dark-red carotenoid pigment (Table BXII.α.107; Poindexter, 1964, 1989; Ballard et al., 1968).

The prosthecate *Brevundimonas* species grow well in peptone–yeast extract media supplemented with salts (Poindexter, 1981, 1989). Although in the literature it is indicated that they generally require a low concentration of organic material for growth, they are able to grow on common, nutrient-rich culture media, like tryptic soy agar or nutrient agar (M. Vancanneyt, personal communication). In standing broth cultures, they may form a pellicle that develops at the surface of the medium and adheres firmly to the wall of the culture vessel (Poindexter, 1989).

The nonprosthecate species, *B. diminuta* and *B. vesicularis*, grow on blood agar, infusion agar, tryptic soy and nutrient agar, Mueller–Hinton medium, and King A medium. After 3 d, a brownish-black, diffusible pigment is produced by most *B. dimi-*

nuta strains. Contrary to Richard and Kiredjian (1995), Gilardi (1978b) has indicated that *B. vesicularis* might occasionally produce a brown discoloration of the agar medium surrounding the colony. *B. diminuta* produces a water-soluble brown pigment. On heart infusion agar with tyrosine, more than 60% of the *B. vesicularis* strains produce a tan-brown, water-soluble pigment, and about 15% of the strains produce a yellow pigment (Gilardi, 1978b; Clark et al., 1984). Most *B. diminuta* strains and only about 25% of the *B. vesicularis* strains grow on MacConkey medium. No growth occurs on *Salmonella–Shigella* agar or on mineral base medium with acetate (Gilardi, 1978b; Gilligan, 1995). Hemolysis is not observed, and the egg-yolk reaction for lecithinase is negative (Gilardi, 1978b).

The optimal growth temperature is 28–30°C. Maximum growth for most species occurs at around 37°C, although nonprosthecate species grow well at this temperature, and about 50% of *B. diminuta* strains are able to grow at 42°C. Suboptimal growth is observed at 25°C, and no growth is observed at 4°C (Ballard et al., 1968; Gilardi, 1978b; Poindexter 1981).

Optimal pH is around neutrality. For the species examined, growth is observed in a range of pH 6–8 (Poindexter, 1964), although strains have been isolated from water and soil with a pH of about 5–9 (Poindexter, 1981).

Although *Brevundimonas* strains can grow without sodium chloride, optimal growth is obtained in a range of 0.5–2% NaCl for all species. Depending on the species, reduced growth may occur with salt concentrations of 2–6% and no growth is obtained at concentrations of 6–8% NaCl (Abraham et al., 1999).

Nutrition All species require growth factors in chemically defined media (Poindexter 1964; Ballard et al., 1968). For the prosthecate species, growth factors (available in peptone or yeast extract) remain undetermined, except for *B. intermedia*, for which growth is supported by biotin (Poindexter, 1981). Both non-

TABLE BXII.α.107. Differentiation of the *Brevundimonas* species from phylogenetically or phenotypically related taxa^{a,b}

Characteristic	<i>Brevundimonas</i>		<i>Mycoplana bullata</i>	<i>Caulobacter</i>		<i>Asticcacaulis</i>	<i>Mycoplana sensu stricto</i> ^f	<i>Pseudomonas</i> (rRNA group I ^g)
	Nonprosthecae species ^c	Prosthecae species ^d		Prosthecae species ^e	<i>Caulobacter segnis</i>			
<i>Cell morphology:</i>								
Rods	+							+
Dimorphic rods		+		+		+		
Irregular rods (branching filaments)			+		+		+	
<i>Prosthecae:</i>								
Polar		+		+				
Subpolar/lateral						+		
None	+		+		+		+	+
<i>Flagellation:</i>								
Peritrichous			+		+		+	
Polar	+ ^h	+ ⁱ		+ ^j				+ ^l
Subpolar						+ ^k		
Pigmentation	C, O, Y	C, P, Y, O	W	C, P, Y, O	W	C	W	C, Fl, Yo
<i>Growth factors:</i>								
Biotin	+	+				+	+	
Cyanocobalamin	+							
Cystine	+							
Panthothenate	+		+					
Riboflavin					+			
Thiamine							+	
Vitamin B ₉ or B ₁₂				+				
Other unidentified growth factors		+	+	+				
Nitrate reduction	Rarely	– or d	Weak or –	– or d	–	d	d	d
<i>Carbon assimilation:</i>								
Normal					+	+	+	+
Normal to restricted		+		+				
Restricted	+		+					
Major polar lipids	PG, PGL, GL, GOL, DGL, SQD	PG, PGL, GL, GOL, DGL, SQD	PG, PGL, GL, GOL, DGL, SQD	PG, PGL, GL, GOL	PG, PGL, GL, GOL	PG, GL, GOL	nd	DPG, PE, PG, PC
Major hydroxy fatty acids	C _{12:0} 3OH	C _{12:0} 3OH	C _{12:0} 3OH	C _{12:1} 3OH	C _{12:0} 3OH, C _{12:1} 3OH, C _{16:1} 2OH	C _{12:1} 3OH	C _{14:0} 3OH	C _{10:0} 3OH, C _{12:0} 3OH
No growth above NaCl (% w/v)	6–8	6–8 ^m	6–8	1–2 ^m	1–2	2–3	nd	nd
Major polyamines	SPD, HOMOSPD	nd	nd	nd	nd	nd	nd	PUT, SPD
Ubiquinone	Q-10	Q-10	Q-10	Q-10	Q-10		Q-10	Q-9
Mol% G + C of DNA	65–68	62–67	66–68	62–67	66–68	55–61	64–65	55–65

^aFor symbols, see standard definitions; nd, not determined; C, colorless; O, orange; Y, yellow; Yo, yellow-orange; P, pink; W, white; Fl, fluorescent; DGL, 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol; DPG, diphosphatidylglycerol; GL, glucosyldiacylglycerol; GOL, glucuronosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethylamine; PG, phosphatidyl glycerol; PGL, phosphatidyl glucosyl diacylglycerol; SQD, sulfoquinovosyl diacylglycerol; SPD, spermidine; HOMOSPD, homospermidine; PUT, putrescine; Q-8–Q-10, ubiquinone with 8–10 isoprene units, respectively.

^bData from references Yamada et al. (1982); Oyaizu and Komagata (1983), Palleroni (1984), Poindexter (1989), Urakami et al. (1990d), Yabuuchi et al. (1990a), Stead (1992), Takeuchi et al. (1993a), and Abraham et al. (1999)

^c*B. alba*, *B. aurantiaca*, *B. bacteroides*, *B. intermedia*, *B. subvibrioides*, and *B. variabilis*.

^d*B. diminuta* and *B. vesicularis*.

^e*C. fusiformis*, *C. henrici*, and *C. vibrioides*.

^f*Mycoplana dimorpha* and *Mycoplana ramosa*.

^gAccording to Palleroni (1984).

^hSingle flagellum with short wavelength.

ⁱSingle flagellum.

^jSingle flagellum.

^kSingle flagellum.

^lFlagella with long wavelength.

^mVaries depending on species.

prosthecae species require pantothenate, biotin, and cyanocobalamin to support growth. *B. diminuta* requires the amino acid cystine, for which methionine may be substituted, resulting in less effective growth (Ballard et al., 1968).

The nutritional spectrum of the genus *Brevundimonas* is very variable and can be used for species differentiation (Table

BXII.α.108). Only a few carbon sources are assimilated by the majority of the isolates: the organic acids acetate, butyrate, fumarate, pyruvate, and succinate; the amino acids glutamate and proline; and the carbohydrates glucose, galactose, maltose, and starch. The latter carbohydrates are not utilized by *B. diminuta*, which is regarded as an alkali-producing species (Gilardi, 1978b)

TABLE BXII.α.108. Differential features among species of the genus *Brevundimonas*^{a,b}

Characteristic	<i>B. diminuta</i> ^{c,d,e}	<i>B. alba</i>	<i>B. aurantiaca</i>	<i>B. bacteroides</i>	<i>B. intermedia</i>	<i>B. subvibrioides</i>	<i>B. variabilis</i>	<i>B. vesicularis</i> ^{c,d,e}
Morphology:								
Cell type:								
Bacteroid	+			+			+	+
Subvibrioid		+				+		
Vibrioid			+		+			
Prostheca (stalk)	—	+	+	+	+	+	+	—
Nonstalked pole tapered				—	+		—	
Stalk invariably central				+	+		—	
Pigmentation	C	C	R, G	C, Y, O	C	O, G	C, Ro	P, Y, O, C
Required growth factors:								
Biotin	+			—	+	—	—	+
Cyanocobalamin	+							+
Cystine	+							—
Pantothenate	+	—	—	—	—	—	—	+
Amino acids				d	—	—	—	
Others, unidentified		+		+	+	+	+	
Assimilation of:								
DL-Arabinose ^f	—	—	—	+	+	—	—	—
D-Cellobiose	—							d(+)
D-Fructose ^f	—	—	—	+	—	—	—	—
D-Galactose ^f	—	+	+	+	+	+	+	+
D-Glucose ^f	—	+	+	+	+	+	+	+
Lactose	—	—	—	d	+	—	—	—
Maltose	—	+	+	d	+	+	+	+
D-Mannose ^f	—	d	—	+	+	—	—	—
L-Rhamnose ^f	—							d(—)
D-Ribose	—	d	—	+	—	—	—	—
Starch	—	+	+	+	+	d	+	d(+)
Sucrose	—	+	+	+	+	+	d	—
DL-Xylose ^f	—	—	—	+	+	+	d	—
D-α-Alanine and L-Alanine ^f	d(—)	+	+	d	+	+	d	d(—)
Casamino acids		+	+	d	+	+	+	—
L-Arginine ^f	d(—)	—	—	—	—	—	—	—
L-Aspartate ^f	+	+	+	d	+	+	—	d(—)
L-Glutamate ^f	+	+	+	d	+	+	+	+
L-Histidine ^f	+	—	—	—	—	—	—	—
L-Isoleucine ^f	d(+)	+	—	—	—	—	—	—
L-Leucine ^f	+	d	—	d	—	d	+	d(—)
L-Proline ^f	+	d	+	d	+	d	+	+
L-Norleucine ^f	d(—)							—
DL-Norvaline ^f	d(+)							d(—)
L-Serine ^f	+	—	+	—	+	d	—	d(—)
L-Threonine ^f	d(+)							d(—)
L-Tyrosine ^f	d(—)	—	—	—	—	—	—	—
L-Valine ^f	d(—)	+	—	—	—	—	—	—
Acetate	+	+	+	+	+	+	d	d(—)
Aconitate	—							d(—)
Butyrate	+	d	+	d	+	d	+	d
Fumarate	d(—)	+	+	+	+	+	+	d(+)
Isovalerate	d(+)							—
2-Ketoglutarate	d(—)							—
DL-Lactate ^f	—	—	+	d	—	—	—	d(+)
L-Malate ^f	d(—)	d	+	d	+	d	—	d(+)
Pimelate	—	—	—	—	+	—	—	—
Succinate	d(—)	+	+	+	+	+	+	d(+)
n-Valerate	d(+)							—
Butanol	—	d	—	—	+	—	d	—
Ethanol		d	+	d	d	—	—	d
Methanol	—	—	—	—	—	—	—	—
Pentanol	—	d	—	—	—	—	—	—
Propanol		d	—	—	—	—	—	—
Enzyme activity:								
Nitrate reduction	—	—	—	d	—	—	—	—
Hydrolysis of esculin	—							+
Starch hydrolysis		—	+	(+)	+	+	+	+
α-Glucosidase	—							+
Valine arylamidase	—							d(—)
Chymotrypsin	d(+)							—

^aFor symbols see standard definitions; C, colorless; R, red; Ro, red-orange; G, golden; Y, Yellow; O, orange; P, pink.^bData from Poindexter (1964, 1981, 1989), Palleroni (1984), Urakami et al. (1990d), Segers et al. (1994), and Abraham et al. (1999).^cReactions of the type strain are given between brackets for *B. diminuta* and *B. vesicularis*.

^dAdditional nutritional features for *B. diminuta* and *B. vesicularis* as determined by API 50CH, 50AO, and 50AA galleries. Substrates used by fewer than 10% of the strains are: adonitol, amygdalin, D-arabitol, L-arabitol, arbutin, dulcitol, erythritol, D-fucose, L-fucose, β-gentiobiose, gluconate, 2-ketogluconate, 5-ketogluconate, N-acetyl-glucosamine, methyl-α-D-glucose, glycerol, glycogen, inositol, inulin, D-lyxose, mannitol, methyl-α-D-mannoside, D-melezitose, D-melibiose, D-raffinose, salicin, sorbitol, L-sorbose, D-tagatose, trehalose, D-turanose, xylitol, methyl-β-xyloside, adipate, azelate, benzoate, m-hydroxybenzoate, o-hydroxybenzoate, p-hydroxybenzoate, caprate, n-caproate, caprylate, citraconate, citrate, isobutyrate, glutarate, DL-glycerate, glycolate, heptanoate, itaconate, levulinate, maleate, D-malate, malonate, D-mandelate, L-mandelate, mesaconate, oxalate, phenylacetate, phthalate, isophthalate, terephthalate, pelargonate, propionate, sebacate, suberate, D-tartrate, L-tartrate, meso-tartrate, acetamide, amylamine, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, benzylamine, betaine, diaminobutane, butylamine, DL-2-aminobutyrate, DL-3-aminobutyrate, DL-4-aminobutyrate, L-citrulline, creatine, L-cysteine, ethanolamine, ethylamine, glucosamine, glycine, histamine, DL-kynurenine, L-norleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, sarcosine, spermine, trigonelline, tryptamine, D-tryptophan, L-tryptophan, urea, and DL-5-aminovalerate. DL-β-hydroxybutyrate is used by all strains of both species. Also, the following substrates are not used by either species (according to conventional test results; Segers et al., 1994): anthranilate, benzoylformate, 2,3-butyleneglycol, n-dodecane, eicosanedioate, ethyleneglycol, geraniol, n-hexadecane, hippurate, isobutanol, isopropanol, kynurenate, methylamine, mucate, naphthalene, nicotinate, phenol, phenylethanediol, propyleneglycol, quinate, saccharate, testosterone and DL-α-aminovalerate. A negative reaction is also obtained by conventional tests (Segers et al., 1994) for gelatin liquefaction, indole formation, urease, lysine and ornithine decarboxylases, arginine dihydrolase, phenylalanine deaminase, lecithinase (egg yolk), and lipase (Tween 80).

^eActivity of the following enzymes as determined for *B. diminuta* and *B. vesicularis* by API ZYM. Always absent: lipase (C₁₄), cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl β-glucosidase, and α-mannosidase; present: alkaline and acid phosphates, ester lipase (C₈), leucine arylamidase, phosphoamidase, trypsin, and indophenol oxidase.

^fIsomer not specified for *B. alba*, *B. aurantiaca*, *B. bacteroides*, *B. intermedia*, *B. subvibrioides*, and *B. variabilis*.

because of its inability to utilize most carbohydrates. Characteristic for both nonprosthecae species is also the utilization of DL- β -hydroxybutyrate, a feature that has not been tested for the prosthecae species. It is clear from Table BXII.108 that several phenotypic characteristics are available solely for either the prosthecae or the nonprosthecae species, which is a result from their—until recently—different generic classification. No comprehensive phenotypic studies are available in which all species of the genus are compared in a consistent and reproducible way.

The nonprosthecae species are oxidase positive and urease and gelatinase negative, and they do not produce indole. The prosthecae species have, as far as is known, not been examined for these features. Metabolic studies using *B. bacteroides* have indicated the presence of 2-keto-3-deoxy-6-phosphogluconate aldolase which suggests that glucose is catabolized via the Entner–Doudoroff pathway (Poindexter, 1981). *Brevundimonas* strains also have the ability to degrade or detoxify aromatic compounds and organophosphates (Poindexter, 1981; Weissenfels et al., 1990; Caldwell et al., 1991; Labuzek et al., 1994; Hoskin et al., 1995; Hong and Raushel, 1996; Davis et al., 1997), a characteristic that has recently been used to develop an amperometric enzyme biosensor for the direct measurement of parathion (Sacks et al., 2000). *B. diminuta* strains are also characterized by a specific control mechanism for DAHP synthetase (3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase), an activity that is not always recovered for *B. vesicularis* (Whitaker et al., 1981). Byng et al. (1980) have demonstrated the presence of an NADP-dependent prephenate dehydrogenase (tyrosine biosynthesis) in nonprosthecae *Brevundimonas* species, and West (1992) has studied their ribonucleoside catabolic enzyme activities. Polyvinyl alcohol oxidase activity has been demonstrated for a particular *B. vesicularis* strain (Kawagoshi and Fujita, 1997; Kawagoshi et al., 1997). EDTA resistance is demonstrated for *B. diminuta* (Wilkinson, 1968). Finally, the unusual metabolic capacities of some *Brevundimonas* members have recently been investigated for bioconversion of intermediate components of cephalosporin production (e.g., Kim et al., 2000b).

Lipid composition The polar lipids are α -D-glucopyranosyl diacylglycerol, α -D-glucopyranuronosyl diacylglycerol, 6-phosphatidyl- α -D-glucopyranosyl diacylglycerol, phosphatidylglycerol, 1,2-di-O-acyl-3-O-[D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranuronosyl] glycerol, and sulfoquinovosyl diacylglycerol (Abraham et al., 1997).

Data on the lipopolysaccharides are available only for the two nonprosthecae species and have some unusual features (Wilkinson and Taylor, 1978). The lipid A contains 2,3-diamino-2,3-dideoxy-D-glucose instead of 2-amino-2-deoxy-D-glucose (the backbone of lipid A in lipopolysaccharides of most Gram-negative bacteria). The component 9-hydroxy- δ -tetradecalactone has been detected in lipid A of both species (Arata et al., 1989). Acetylated mannan and D-threo-pent-2-ulose have been isolated from the lipopolysaccharides of one *B. diminuta* strain NCTC 8545 (Wilkinson 1981b, a). Purified lipid A preparations of *B. vesicularis* may activate macrophages to resist infection by an opportunistic bacterium in mice (Arata et al., 1994).

Brevundimonas strains are characterized by the following dominant fatty acids C_{12:0} 3OH (1–3%), C_{14:0} (tr–5%), C_{15:0} (tr–8%), C_{16:0} (10–24%), C_{16:1} (1–11%), C_{17:0} (tr–8%), C_{17:1} ω 6c (tr–6%), C_{17:1} ω 8c (1–11%), and C_{18:1} (39–69%) (Abraham et al., 1999). Species may be differentiated on the basis of quantitative differ-

ences in these fatty acids. *B. diminuta* and *B. alba* are further differentiated from the other *Brevundimonas* species by the presence of a significant amount of C_{19:0} cyclo ω 8c (methyleneoctadecanoic acid; Yamada et al., 1982; Segers et al., 1994; Abraham et al., 1997, 1999).

Polyamines Prosthecae species have not been examined for the presence of polyamines. *B. diminuta* strains produce homospermidine as the major polyamine component (1.7–3.4 μ mol/g protein) as well as trace amounts of spermidine. *B. vesicularis* strains (except one strain) produce spermidine as the major component (1.6–2.5 μ mol/g protein), homospermidine as a minor component (0.1–0.5 μ mol/g protein), and, in some cases, trace amounts of norspermidine, putrescine, and spermine. Two currently unnamed *Brevundimonas* strains yield a similar polyamine pattern to the *B. vesicularis* strains, and another strain produces only spermidine in high amounts (Segers et al., 1994).

Genetic features Rather small genomes (3–3.5 Mb) are observed for the nonprosthecae species (Grothues and Tümmeler, 1991). No information on genome size is available for the prosthecae species. Plasmids have so far been detected only for *B. bacteroides* (Poindexter, 1989). DNA–DNA hybridization studies among all currently described species of *Brevundimonas* (M. Vancanneyt, personal communication) have shown relatedness values below 70%, thus confirming their status as separate species. Intermediate levels of DNA–DNA relatedness of 50% and 41% are observed between the nonprosthecae species *B. vesicularis* and the prosthecae species *B. aurantiaca* and *B. intermedia*, respectively. The latter two species show 65% DNA–DNA relatedness, indicating their close relationship.

Antimicrobial agents All species are sensitive to penicillin G and streptomycin. Significantly more data on antibiotic resistance are available for the two nonprosthecae species, which may be explained by their occurrence in clinical sources. *B. diminuta* is sensitive to aminoglycosides and β -lactam antibiotics and is highly sensitive to tetracyclines and rifampicin. Susceptibility to carbenicillin, sulfonamides, and chloramphenicol differs among strains of the species. All strains are resistant to nalidixic acid (a diagnostic feature), furans, trimethoprim, colistin, ampicillin, cephalothin, polymyxin, and gentamicin. *B. vesicularis* is usually more sensitive to antibiotics than is *B. diminuta*, especially to cephalothin (Gilardi, 1976; Richard and Kiredjian, 1995); however, treatment with tobramycin and ceftazidime of a case of *B. vesicularis* bacteremia in a patient was not successful (Planes et al., 1992).

B. subvibrioides and *B. bacteroides* have been found to be sensitive to some *Caulobacter* phages (Poindexter, 1989).

Source or habitat The organisms are typical aquatic bacteria. At least some species have been isolated from mineral water (Jayasekara et al., 1999). Prosthecae species may be isolated from soil (e.g., *B. alba*). Nonprosthecae species have also been identified in clinical specimens, such as blood cultures, infected biological fluids, urine, wounds, vagina, eye, and tissue cultures (Aspinall and Graham, 1989; Morais and da Costa, 1990; Gilardi, 1991; Planes et al., 1992; Abraham et al., 1997; Giardini et al., 1997). In particular, *B. vesicularis* has been reported as an important nosocomial agent (Gilad et al., 2000).

ENRICHMENT AND ISOLATION PROCEDURES

There is no strong selective enrichment procedure described for the isolation of *Brevundimonas* strains. Nevertheless, the tolerance

of prolonged nutrient scarcity by prosthecae members of the genus provides a dependable physiological basis for their enrichment. Successful enrichments from water and from soil suspended in water are obtained when the sample is allowed to stand undisturbed for one to several weeks (Poindexter, 1992). Enrichment/isolation procedures are also described based on the accumulation of the stalked cells at the air–water interface or on a cover slip of a liquid culture with low nutrient content that is carefully protected against turbulence (Houwink, 1955; Poindexter, 1989). Methods and media for enrichment and further differentiation from other organisms are described in more detail in this chapter and in the literature (Leifson and Hugh, 1954b; Poindexter, 1964, 1981, 1989, 1992; Ballard et al., 1968; Gilardi, 1978b; Clark et al., 1984; Palleroni, 1984; Segers et al., 1994; Gilligan, 1995; Grimont et al., 1996).

MAINTENANCE PROCEDURES

Strains can be easily cultured aerobically on most common agar media containing peptone or yeast extract at 30°C. These media support growth without growth-factor supplements, which are necessary in defined media and enhance growth in complex media (Table BXII.α.107). Nonprosthecae cultures can be maintained for at least 1 week at room temperature or in a refrigerator before being transferred. Prosthecae isolates streaked on dilute complex media (and incubated for 2 or 3 d at 20–30°C) should be transferred every 8–9 weeks when refrigerated (Poindexter, 1989).

Cultures may be stored for many years by lyophilization, by freezing at –80°C, or in liquid nitrogen. In general, cryoprotective agents such as 10% glycerol or dimethyl sulfoxide are added to cultures before freezing, although prosthecae species can be frozen at –70°C without cryoprotectant (Poindexter, 1989).

DIFFERENTIATION OF THE GENUS *BREVUNDIMONAS* FROM OTHER GENERA

Characteristics that differentiate *Brevundimonas* from phylogenetically and phenotypically related genera and species are listed in Table BXII.α.107. *Caulobacter* is the closest phylogenetic neighbor with 16S rDNA sequence similarities of 95–96%. Based on morphological features, both nonprosthecae species of *Brevundimonas* are easily distinguished from *Caulobacter* species of which all, except *Caulobacter segnis*, are stalk-forming organisms. Within the latter genus, *C. segnis* has a unique morphology with irregular, peritrichously flagellated rods and branching filaments. Morphology does not allow differentiation among prosthecae species of both taxa. There are also no straightforward nutritional properties that unequivocally distinguish both genera, although particular tests can be used to characterize individual species (Table BXII.α.108; Poindexter, 1989). Some useful cultural and chemotaxonomic differentiating features between the genera *Brevundimonas* and *Caulobacter* are their salt tolerances and polar lipid and fatty acid contents (Table BXII.α.107; Stahl et al., 1992; Abraham et al., 1999). Strains of the genus *Caulobacter* show optimal growth at 0.5% NaCl and reduced or no growth at salt concentrations of 1–2% (depending on the species). *Brevundimonas* strains have a broader optimal salt concentration of 0.5–2% NaCl. The polar lipids 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol, and sulfoquinovosyl diacylglycerol are present in most *Brevundimonas* strains (>80%) and absent in

Caulobacter. *Brevundimonas* is furthermore differentiated from *Caulobacter* by the absence of significant amounts of C_{12:1 3OH} and of an unknown fatty acid with ECL 11.789, and by the presence of at least traces of an unknown fatty acid with ECL 17.897 and higher amounts of C_{12:0 3OH} and C_{18:1} (Abraham et al., 1999).

Differentiation among *Brevundimonas* and *Caulobacter* strains is also obtained based on the reactivity of antisera. A 43-kDa protein exhibits genus-specific epitopes (Abraham et al., 1999). These authors have also demonstrated that desorption chemical ionization (DCI) mass spectrometry of glycolipids and subsequent multivariate analysis of the relative intensities of the [M+NH₄]⁺ ions between m/z 740–840 has allowed differentiation between the genera *Brevundimonas* and *Caulobacter*.

Asticcacaulis is, at the generic level, the closest phylogenetic neighbor of *Brevundimonas* and *Caulobacter*, but clearly represents a separate branch within the family *Caulobacteraceae* (16S rDNA sequence similarity of 93–94%; Abraham et al., 2001). The genus *Asticcacaulis* was created by Poindexter (1964) to accommodate prosthecae isolates originally regarded as *Caulobacter* species. They are morphologically distinguished from prosthecae *Brevundimonas* and *Caulobacter* species by the site of adhesion of sessile cells to substrata; *Brevundimonas* and *Caulobacter* cells adhere by the distal tip of the prosthecae, while *Asticcacaulis* cells adhere by the cell pole, and the prosthecae is not adhesive (Poindexter, 1989, 1992). With a mol% G + C content between 55 and 61, *Asticcacaulis* can be differentiated from *Brevundimonas* and *Caulobacter* by its mol% G + C content of 62–68. The phylogenetic branches are further differentiated by the lack of 1,2-diacyl-3-*O*-[6'-phosphatidyl-α-D-glucopyranosyl]glycerol (PGL) in *Asticcacaulis*. Strains of this genus also do not contain 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol (DGL) found in most *Brevundimonas* strains but not in strains of the genus *Caulobacter* (Abraham et al., 2001). These authors have demonstrated, furthermore, that *Asticcacaulis* species are characterized by the presence of C_{12:1 3OH} in their fatty acid profile, a component that has also been observed to be characteristic for *Caulobacter* species, but not for *Brevundimonas* taxa.

A deeper branching in the *Caulobacteraceae* is found for *Phenyllobacterium*, a taxon characterized by nonmotile rods or cocci that do not produce prosthecae. Although there are some common features with the other members of the family, such as their growth factor requirements and carbohydrates for growth, the genus is easily distinguished by its high nutritional specialization. Good growth occurs on chloradizon, antipyrin, and L-phenylalanine (Lingens et al., 1985).

The two nonprosthecae species of *Brevundimonas* were previously classified in *Pseudomonas* (Palleroni, 1984). Both species can easily be differentiated from members of the present genus *Pseudomonas* (in general terms restricted to rRNA group 1; Palleroni, 1984) not only by their separate phylogenetic position, but also by one or more of the following characteristics: cell morphology, short wavelength of the flagella, polyamines and quinone content, a restricted nutritional spectrum, and their salt tolerance (Table BXII.α.107; Ballard et al., 1968; Gilardi, 1978b; Palleroni, 1984; Segers et al., 1994; Abraham et al., 1997, 1999). Both species are further distinguished by the absence of phosphatidylethanolamine and by the presence of phosphatidylglycerol, phosphatidylglucosyldiacylglycerol, and some major glycosyldiacylglycerols in their polar lipids (Wilkinson and Bell, 1971; Wilkinson and Taylor, 1978; Wilkinson and Galbraith, 1979;

Barnes et al., 1989). The presence or absence of specific 2-OH and 3-OH fatty acids can be used as diagnostic features to differentiate *Brevundimonas* taxa from other members of the former genus *Pseudomonas* (Table BXII.α.107; Oyaizu and Komagata, 1983; Palleroni, 1984; Segers et al., 1994). Also, genomic differences are found, like the small genomes of *Brevundimonas* spp. (3–3.5 Mb) and the absence of host factor for coliphage Qβ RNA replication (DuBow and Ryan, 1977; Grothues and Tümmeler, 1991).

TAXONOMIC COMMENTS

The genus *Brevundimonas* currently contains eight species and belongs to the class *Alphaproteobacteria* as a member of the family *Caulobacteraceae*. Originally, *Brevundimonas* contained only *B. diminuta* and *B. vesicularis*, which were reclassified members of the genus *Pseudomonas sensu lato* (Segers et al., 1994). Techniques based on rRNA similarities (Palleroni et al., 1973; De Vos and De Ley, 1983) demonstrated the phylogenetic heterogeneity of the latter genus and showed that the rRNA Group IV members, *Pseudomonas diminuta* and *Pseudomonas vesicularis*—often called the “diminuta group” (Ballard et al., 1968; Gilardi, 1978b)—were found to occupy a unique position within the *Alphaproteobacteria*. It was also shown by Segers et al. (1994) that some strains that were preliminarily classified into EF group 21, a heterogeneous group containing organisms that show phenotypic similarities to *Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*), are members of *Brevundimonas*.

In 1999, 16S rRNA gene sequencing studies indicated that *B. vesicularis* and *B. diminuta* are phylogenetically interrelated with *Caulobacter intermedius* and *Caulobacter variabilis* (Abraham et al., 1999; Sly et al., 1999) and with *Caulobacter subvibrioides* and *Caulobacter bacteroides*, as well as with the misclassified *Mycoplana bullata* (Abraham et al., 1999). Other validly described species of the genus *Caulobacter*, including the type species *Caulobacter vibrioides* and *Caulobacter segnis*, constitute a separate phylogenetic branch within the family *Caulobacteraceae* (Abraham et al., 1999; Fig. BXII.α.122).

A formal reclassification has been made based on phylogenetic data and the genus extended with six new species, *B. bacteroides*, *B. intermedia*, *B. subvibrioides*, *B. variabilis*, *B. alba*, and *B. aurantiaca*. The latter two were originally described as *C. subvibrioides* subsp. *alba* and *C. henricii* subsp. *aurantiaca*, respectively. Based on their distinct phylogenetic position in the genus, they have been elevated to the species rank (Abraham et al., 1999).

Abraham et al. (1999) have also emended the genus description of *Caulobacter*, which currently contains, in addition to the type species *Caulobacter vibrioides*, the species *Caulobacter fusiformis*, *Caulobacter henricii*, *Caulobacter leidy*, and *Caulobacter segnis*. *Cau-*

lobacter segnis was originally named *Mycoplana segnis*, a misclassified member of *Mycoplana* that belongs to a different lineage in the *Alphaproteobacteria* within the family *Rhizobiaceae*. Furthermore, Abraham et al. (1999) have proposed that *Caulobacter crescentus* is a subjective synonym of *C. vibrioides*.

As shown in Fig. BXII.α.122, not only *Caulobacter segnis* (*Mycoplana segnis*) was misclassified, but also *Mycoplana bullata*, which clearly belongs on phylogenetic arguments to *Brevundimonas*. Additionally, 39% DNA relatedness has been found to the type strain of *B. diminuta* by DNA–DNA hybridizations (M. Vancanneyt, personal communication). Up to now, however, no formal reclassification has been proposed for the misclassified *Mycoplana bullata*. Except for the very distinct morphologic characteristics, i.e., peritrichously flagellated, rod-shaped cells forming filaments before fragmentation, most genomic, chemotaxonomic and nutritional features of *Mycoplana bullata* are analogous to those of related species of *Brevundimonas* (Table BXII.α.107; Yanagi and Yamasato, 1993; Abraham et al., 1999). If a formal reclassification is proposed to include *M. bullata* as a member of *Brevundimonas*, the emended genus description of the latter will have to encompass the features of *Mycoplana bullata*, as indicated below.

FURTHER READING

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *BREVUNDIMONAS*

Features for differentiating *Brevundimonas* species are the cell morphology and cell division, stalk formation, pigmentation, re-

quirement for growth factors, nutritional spectrum, and other characteristics as shown in Table BXII.α.108.

List of species of the genus *Brevundimonas*

1. ***Brevundimonas diminuta*** (Leifson and Hugh 1954b) Segers, Vancanneyt, Pot, Torck, Hoste, Dewettinck, Falsen, Kersters and De Vos 1994, 507^{VP} (*Pseudomonas diminuta* Leifson and Hugh 1954b, 68.)
di.mi.nu'ta. L. adj. *minutus* small; M.L. fem. adj. *diminuta* defective, minute.

Short rods. Motile by means of one polar flagellum that has a short wavelength (0.6–1 μm). No prosthecae or special cell division. No pigmentation. The description of *B. diminuta* is the same as for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

Isolated from water, aqueous solutions and diverse clinical specimens in man and animals.

The mol% G + C of the DNA is: 66–68 (T_m).

Type strain: ATCC 11568, DSM 7234, IMET 10409, LMG 2089.

GenBank accession number (16S rRNA): AJ227778.

2. **Brevundimonas alba** (ex Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1070^{VP} *al'ba*. L. fem. adj. *alba* white.

Cell morphology varies within a clone; some poles distinctly tapered, others rounded; long axis gently curved. Colonies colorless. The description of *B. alba* is the same as that for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

Strains are characterized by the presence of detectable amounts of $C_{19:0\text{ cyclo } \omega 8c}$.

The type strain was isolated from soil.

The mol% G + C of the DNA is: 67 (Bd); 68 (HPLC).

Type strain: CB88, DSM 4736, LMG 18360.

GenBank accession number (16S rRNA): AJ227785.

3. **Brevundimonas aurantiaca** (ex Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1071^{VP} *au.ran.ti.a'ca*. M.L. fem. adj. *aurantiaca* orange.

Vibrioid, unusually small cells. Dark golden colonies. The description of *B. aurantiaca* is the same as that for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

The type strain was isolated from a contaminated *Chlorella* culture.

The mol% G + C of the DNA is: 67 (HPLC).

Type strain: CB-R, DSM 4731, LMG 18359.

GenBank accession number (16S rRNA): AJ227787.

4. **Brevundimonas bacteroides** (Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1071^{VP} (*Caulobacter bacteroides* Poindexter 1964, 272.)

bac.ter.oi'des. M.L. *bacter* masc. form of Gr. neut. n. *bactrum* rod; Gr. n. *eidus* form, shape; M.L. adj. *bacteroides* rod shaped.

Slender, rod-shaped cells. Stalk typically has a bulbous distal tip. Colonies colorless or brightly pigmented yellow or orange. The description of *B. bacteroides* is the same as that for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

Isolated from water and soil.

The mol% G + C of the DNA is: 66 (Bd); 68 (HPLC).

Type strain: CB7, ATCC 15254, DSM 4726, LMG 15096.

GenBank accession number (16S rRNA): AJ227782.

5. **Brevundimonas intermedia** (Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1071^{VP} (*Caulobacter intermedius* Poindexter 1964, 272.)

in.ter.med'i.a. L. adj. *intermedia* in the middle degree, between extremes.

Short, vibrioid cells. Colorless colonies. The description of *B. intermedia* is the same as that for the genus, with the

additional characters given in Table BXII.α.107 and BXII.α.108.

Isolates reported from pond water.

The mol% G + C of the DNA is: 66 (HPLC).

Type strain: CB63, ATCC 15262, DSM 4732, LMG 18361.

GenBank accession number (16S rRNA): AJ227786.

6. **Brevundimonas subvibrioides** (Poindexter 1964) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1071^{VP} (*Caulobacter subvibrioides* Poindexter 1964, 272.)

sub.vib.ri.oi'des. L. pref. *sub* almost, somewhat, near; M.L. n. *vibrio* name of a genus; Gr. n. *eidus* resembling; M.L. adj. *subvibrioides* somewhat like a vibrio.

Cell morphology variable within a clone: some poles distinctly tapered, others rounded; long axis gently curved or not curved. Colonies orange, golden, or colorless. The description of *B. subvibrioides* is the same as that for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

Isolated from water and soil.

The mol% G + C of the DNA is: 67 (Bd); 68 (HPLC).

Type strain: CB81, ATCC 15264, LMG 14903.

GenBank accession number (16S rRNA): AJ227784.

7. **Brevundimonas variabilis** (Poindexter 1989) Abraham, Strömpl, Meyer, Lindholst, Moore, Christ, Vancanneyt, Tindall, Bennasar, Smit and Tesar 1999, 1071^{VP} (*Caulobacter variabilis* Poindexter 1989, 495.)

vari.i.d'bil.is. M.L. adj. *variabilis* variable.

Short, thick, rod-shaped cells; position of stalk variable, arising from the center of the cell pole or from a subpolar (eccentric) site. Colonies red-orange or colorless. The description of *B. variabilis* is the same as that for the genus, with the additional characters given in Table BXII.α.107 and BXII.α.108.

Isolated from water and soil.

The mol% G + C of the DNA is: 65 (HPLC).

Type strain: CB17, ATCC 15255, DSM 4737, LMG 18362.

GenBank accession number (16S rRNA): AJ227783.

8. **Brevundimonas vesicularis** (Büsing, Döll and Freytag 1953) Segers, Vancanneyt, Pot, Torck, Hoste, Dewettinck, Falsen, Kersters and De Vos 1994, 508^{VP} (*Pseudomonas vesicularis* (Büsing, Döll and Freytag 1953) Galarneault and Leifson 1964, 167; "*Corynebacterium vesiculare*" Büsing, Döll and Freytag 1953, 76.)

ve.si.cu.la'ris. M.L. adj. *vesicularis* pertaining to a vesicle.

Short rods. Motile by means of one polar flagellum that has a short wavelength (0.6–1 μm). No prosthecae or special cell division. An intracellular carotenoid pigment (pink, yellow, or orange) is usually produced. The description of *B. vesicularis* is the same as that for the genus with the additional characters given in Table BXII.α.107 and BXII.α.108.

Isolated from water, aqueous solutions, and diverse clinical specimens from humans and animals.

The mol% G + C of the DNA is: 65–66 (T_m).

Type strain: ATCC 11426, CCUG 2032, DSM 7226, LMG 2350.

GenBank accession number (16S rRNA): AJ227780.

Other Organisms

1. *Mycoplana bullata* Gray and Thornton 1928, 83^{AL}
bul.la'ta. L. adj. *bullata* with a knob.

As shown above and reported in the literature (Fig. BXII.α.122; Abraham et al., 1999), *M. bullata* can also be phylogenetically considered a member of the genus *Brevundimonas*.

Cells are rod-shaped with round ends, occurring singly or rarely in pairs, motile by means of peritrichous flagella. They form branching filaments prior to fragmentation. No stalk formation. Calcium pantothenate and an unidentified compound are required for growth. Nitrate reduction and catalase activity are weak or negative. The Voges–Proskauer

test is positive. Acid is produced oxidatively from D-glucose but is not produced from D-arabinose, D-xylose, D-mannose, D-fructose, D-galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, or soluble starch. Other features are given in Table BXII.α.107 and in Urakami et al. (1990d).

Isolated from soil.

The mol% G + C of the DNA is: 66–68 (T_m).

Deposited strain: ATCC 4278, DSM 7126, IFO 13290, LMG 17157.

GenBank accession number (16S rRNA): D12785.

Genus IV. *Phenylobacterium* Lingens, Blecher, Blecher, Blobel, Eberspächer, Fröhner, Görisch, Görisch and Layh 1985, 38^{VP}

JÜRGEN EBERSPÄCHER

Phe.ny.lo.bac.te'ri.um. Gr. n. *phen* benzene; Gr. n. *bacterion* a small rod; M.L. neut. n. *Phenylobacterium* benzene bacterium, so named because of its unique preference for phenyl moieties from heterocyclic compounds as carbon sources.

Rods, coccobacilli, or cocci; 0.7–1.0 × 1.0–2.0 μm occurring singly, in pairs, or in short chains. Some strains tend to clump. In old cultures, pleomorphic forms, such as long rods (1.0 × 2.0–4.0 μm), long chains (10–50 μm) connected by filaments, and elliptical forms, may occur. Capsule stain negative; flexible capsule present. **Nonmotile**. Nonsporeforming. Non-acid-fast. Sheaths and prosthecae not produced. Nonpigmented. **Gram negative**. Growth on chloridazon–mineral salts agar is slow and colonies are small (1–2 mm) after 2–3 weeks. Colonies may be either smooth, convex, moist with shiny surfaces and entire edges, and easily emulsified in saline, or rough, dry, and not emulsifiable in saline. In liquid media, there is slight production of a greenish-yellow nonfluorescent pigment; in media containing L-phenylalanine a yellowish-green fluorescent pigment is produced. **Strictly aerobic**. **Catalase positive, weakly oxidase positive**. **Chemoorganotrophic, having a strictly respiratory type of metabolism**. Optimal temperature, 28–30°C. No growth at 4°C or 37°C; at 37°C cultures die within several days. Optimal pH 6.8–7.0; growth occurs between pH 6.5 and 8; no growth at pH 4 or 9. **Osmotically sensitive**. **Vitamin B₁₂ is a growth factor**. **High nutritional specialization; grows well on chloridazon, antipyrin, and L-phenylalanine (most strains only after long lag phase)**. Slow growth on L-glutamate, pyruvate, fumarate, succinate, and malate as well as on diluted complex media (0.5–2 g/l peptone). **Most sugars, alcohols, amino acids, carboxylic acids, and ordinary complex media are not utilized**. Does not denitrify; does not produce nitrite from nitrate. NH_4^+ and NO_3^- are used as sole sources of nitrogen; no growth with N_2 . Gelatin, casein, starch, and esculin not hydrolyzed. Urease negative. Litmus milk negative. Weak H_2S production from thiosulfate or cysteine. Methyl red and Voges–Proskauer negative. Indole negative. No acid or gas produced from sugars or alcohols. Not pathogenic for rats or rabbits. **Isolated from soil after enrichment in mineral salts media containing chloridazon, antipyrin, or pyrimidon** (synthetic heterocyclic compounds).

The mol% G + C of the DNA is: 65–68.5.

Type species: *Phenylobacterium immobile* Lingens, Blecher, Blecher, Blobel, Eberspächer, Fröhner, Görisch, Görisch and Layh 1985, 38.

FURTHER DESCRIPTIVE INFORMATION

Two strains of *Phenylobacterium immobile*, namely strains E and A13 (see Fig. BXII.α.123, parts a and b) form exclusively coccobacilli, which tend to clump, and growth in liquid cultures is sometimes flocculent. All other strains have short rod-shaped cells, which occur singly, in pairs, or in short chains. These strains never clump in liquid cultures. Chains are found mainly in cultures of strains that form smooth colonies on agar plates (e.g., strain K₂, see Fig. BXII.α.123c). In old cultures, especially when the bacteria are cultivated in a dilute complex medium that allows only poor growth, pleomorphic forms, such as long rods, long chains of cells connected by small filaments, or club-shaped and elliptical forms, sometimes occur.

Capsule staining and Gram staining are negative. Electron microscopy of thin sections reveal the typical Gram-negative cell wall pattern. Ultrathin sections of ruthenium red-treated cells of strain K₂, which forms smooth colonies, show a microcapsule surrounded by a slime layer of acidic polysaccharides (Fig. BXII.α.124a). No slime layer is detected in ruthenium-red-stained cells of strain E, which forms rough colonies on agar (Fig. BXII.α.124b). According to Costerton et al. (1981), *Phenylobacterium* cells possess flexible capsules, which can be made visible only by electron microscopy, but not rigid capsules, which represent the typical India ink-excluding capsule type.

The murein main component from the type strain is identical to the C₆ muropeptide of *E. coli*. The carbohydrate moiety from the lipopolysaccharide of the type strain consists of heptose, 3-deoxyoctulosonic acid, and D-glucose in a molar ratio of 1:2:2.3 (Weisshaar and Lingens, 1983). Lipid A is composed of 1 mol 2,3-diamino-2,3-dideoxy-D-glucose, 2 mol amide-bound fatty acids, and 2.6 mol ester-bound fatty acids per mol lipid A. 2,3-diamino-2,3-dideoxy-D-glucose is typical of *Alphaproteobacteria* but absent in other Gram-negative bacteria, which contain glucosamine as the main lipid A component. Amide-bound fatty acids are 3-hydroxydodecanoic acid and 3-hydroxyhexadecanoic acid; ester-linked fatty acids are dodecanoic acid and 3-hydroxy-5-*cis*-dodecenoic acid. The detection of the latter, unusual fatty acid, not found in nature before, can be used to demonstrate the presence of *Phenylobacterium immobile* in soil samples (Bellmann and Lingens, 1985).

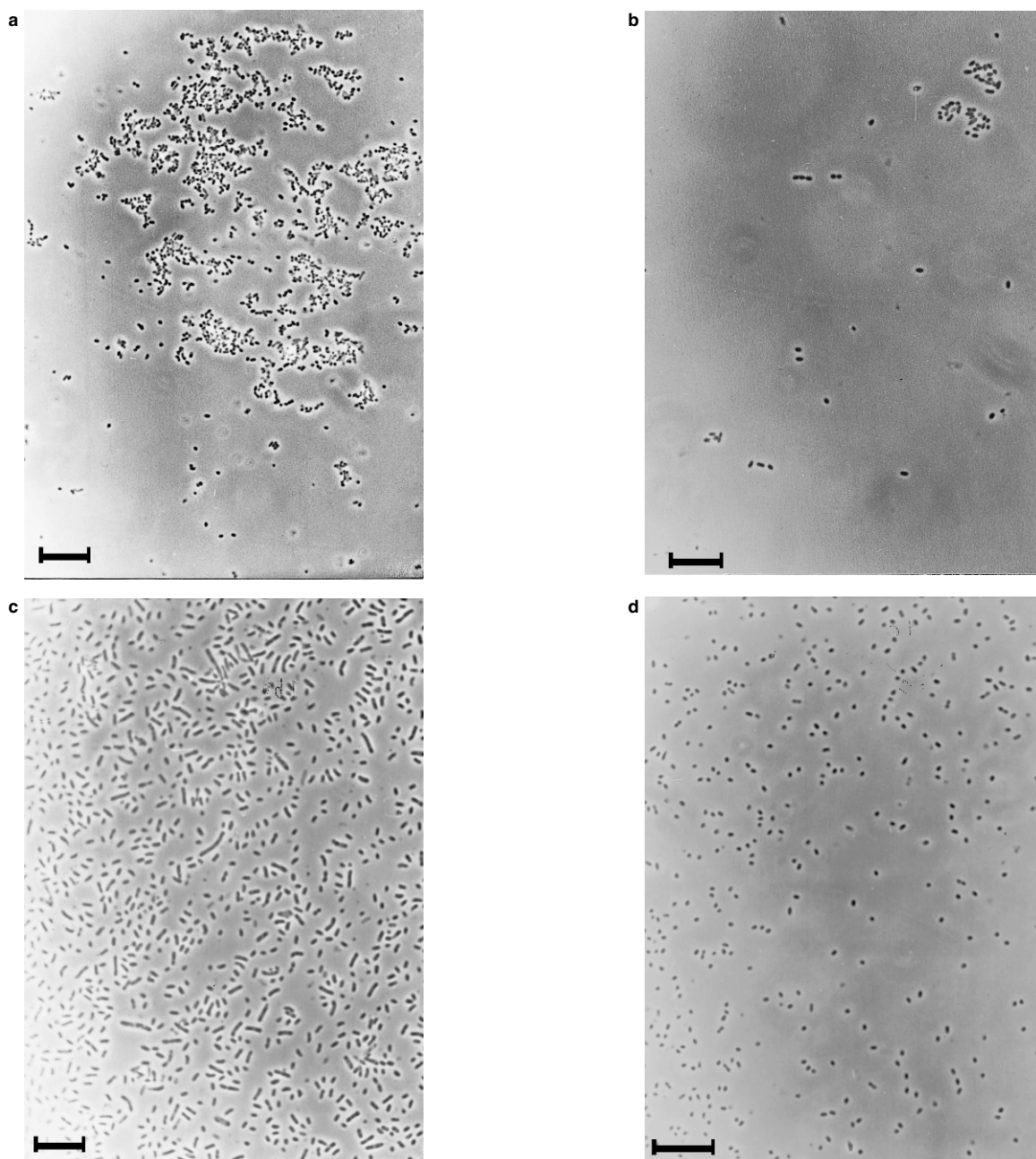


FIGURE BXII.α.123. Phase contrast photomicrographs of cells of *Phenylobacterium immobile*. a) Strain A₁₃; b) strain E, the type strain; c) strain K₂; and d) strain N. Bars = 10 μm.

In liquid cultures, during growth on mineral salts medium with phenylalanine, especially at higher concentrations (3–5 g/l), a yellowish-green fluorescent pigment is produced. On chloridazon or antipyrin mineral salts media, a greenish-yellow non-fluorescent pigment is formed.

Optimal growth on agar plates occurs on mineral salts medium containing chloridazon, antipyrin, or L-phenylalanine as the carbon source. Single colonies are not visible before 4–7 days, and after 2–3 weeks the colonies are 1–2 mm in diameter, circular with entire edges, slightly raised, and not adherent to the agar. Nearly half of the strains form smooth and shiny colonies that can be readily emulsified in water. The other strains have rough and dry colonies that clump when suspended in water.

Each different strain of *Phenylobacterium immobile* does not utilize all three xenobiotic substrates (formula, Fig. BXII.α.125). Whereas chloridazon and antipyrin are well utilized by most strains, only 7 of the 22 isolates use pyrimidon as a growth substrate (Table BXII.α.109). When pyrimidon is added to media containing chloridazon or antipyrin, the growth of all isolates is inhibited.

The pathway for the degradation of the three xenobiotics follows the well-known route for the oxidative dissimulation of aromatic compounds. From these xenobiotic compounds, only the phenyl moiety is used as a carbon source; the heterocyclic moiety remains unchanged. In the first step, O₂ is incorporated in the benzene nucleus by the action of a dioxygenase. The

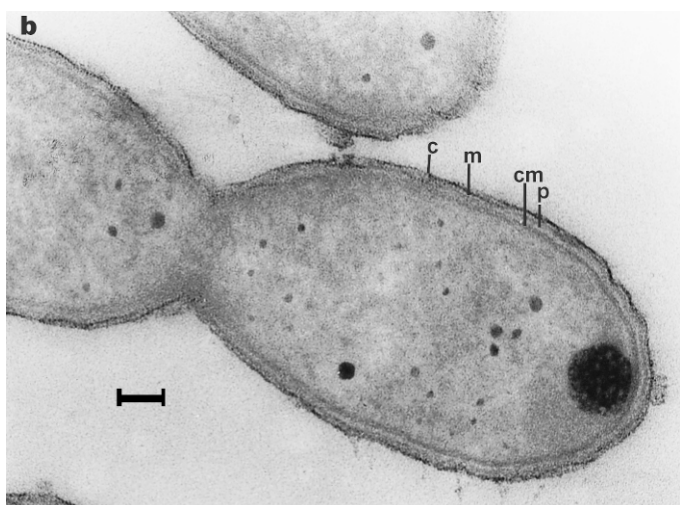
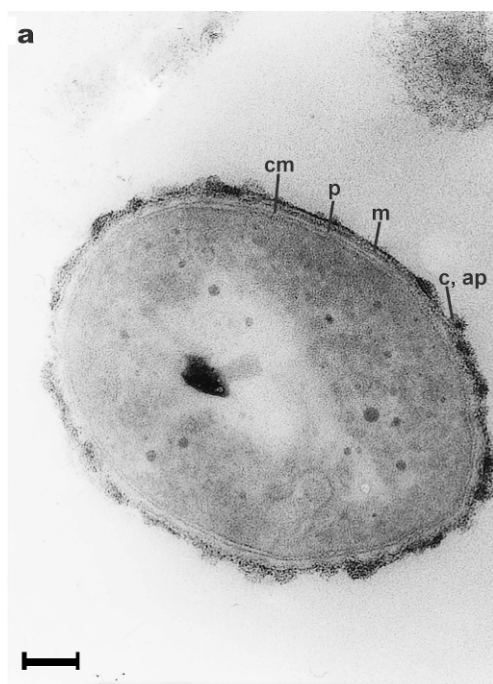


FIGURE BXII.α.124. Ultrathin sections of ruthenium red-treated cells of *Phenyllobacterium immobile*. a) strain K₂; b) strain E^T. cm, cytoplasmic membrane; p, periplasm; m, outer membrane; c, capsule (not stained); c, ap, capsule consisting of acid polysaccharides (stained with ruthenium red).

resulting *cis*-dihydro-dihydroxy compound is oxidized by a dehydrogenase, yielding a catechol derivative, which leads to a dephenylated heterocycle and 2-hydroxymuconate by *meta*-cleavage and the subsequent action of an amidase. Meta-cleaving enzymes from *P. immobile* form a distinct group among nonheme-iron dioxygenases (Schmitt et al., 1984). 2-Hydroxymuconate is further converted via 2-oxo-4-hydroxyvalerate to pyruvate and acetaldehyde, two compounds of intermediary metabolism that are potential carbon sources for the bacterium.

Citric acid cycle enzymes and enzymes catalyzing anaplerotic routes are present in *P. immobile* strain E. Tyrosine is synthesized exclusively via the arogenate pathway, not via prephenate (Keller et al., 1982).

More than 20 different heterocyclic or aromatic compounds that are structurally related to chloridazon or antipyrin have been

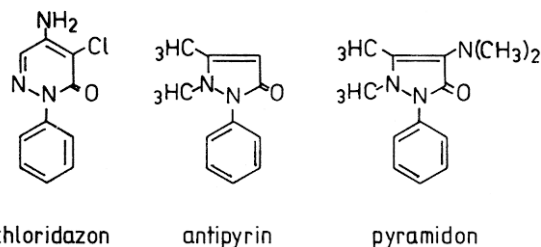


FIGURE BXII.α.125. The herbicide chloridazon and the structurally related analgesics antipyrin and pyramidon were used for the enrichment of *Phenyllobacterium immobile* from soil. These xenobiotics are also the best growth substrates for the bacteria.

tested as possible carbon sources. Most chloridazon analogs with an altered heterocyclic moiety are good growth substrates. A substitution at the aromatic nucleus, as for *o*-, *m*-, or *p*-methylchloridazon, makes the compound nondegradable. Of the aniline derivatives tested, *N*-methylacetanilide and *N*-methylformanilide are poor growth substrates. The following compounds fail to support growth: benzene, toluene, phenol, catechol, benzaldehyde, benzoate, and a number of mono- and dihydroxylated benzoates. One strain (strain N) grows well on L-phenylalanine with a normal lag phase of 1 day; all other strains have lag phases of 2–3 weeks but then grow well on phenylalanine. The long lag phases are observed only for the first transfer of the strains from chloridazon or antipyrin to phenylalanine; thereafter the bacteria grow immediately. Phenylalanine-induced cells also grow well on phenylpropionate, phenylpyruvate, and phenyllactate.

All strains tested are susceptible to tetracycline, bacitracin, chloramphenicol, kanamycin, and streptomycin. The majority (18 of 22) are susceptible to penicillin G and 15 of 22 strains are susceptible to novobiocin. All are resistant to cephaloridine, cloxacillin, fusidic acid, lincomycin, methicillin, and sulfafurazol.

P. immobile strains were found to be harmless when tested in rats and rabbits (Kaiser et al., 1981). No adverse reactions were observed when cells of strains E and N, respectively, were orally administered to rats over a period of 7 d. Exposure via air in inhalation experiments did not lead to specific pulmonary changes. Intracutaneous injection did not cause adverse skin reactions, and intraperitoneal injections did not kill the rats, although bacteria entered the blood. Intravenous injections of living and formalin-inactivated bacteria into rabbits during immunization did not lead to toxic effects.

As shown in Table BXII.α.109, depending on the strain, between one and six different plasmids have been found, which vary in size from 8–300 mDa (Kreis et al., 1981).

Agglutination and immunofluorescence tests with antisera against 4 strains of *Phenyllobacterium immobile* have revealed the serological uniformity of the different strains (Layh et al., 1983). Slight differences in immune reactions allow a classification of the strains into 5 serological subgroups. No relationship was found between *Phenyllobacterium immobile* and 40 representative Gram-negative bacteria, including *Acinetobacter calcoaceticus*, *Azospirillum brasilense*, *Caulobacter* spp., *Paracoccus denitrificans*, some *Pseudomonas* spp., *Rhizobium* spp., *Rhodocyclotrichum vanniellii*, and *Rhodospseudomonas capsulata*. A slight but significant immunofluorescence reaction has been observed with *Brevundimonas vesicularis*, *Gluconobacter oxydans*, *Aquaspirillum itersonii*, and *Rhodospirillum rubrum* (Dorfer et al., 1985). Crossed immunoelectrophoresis reveals a serological relationship between *Phenyllobacterium immobile* and *Brevundimonas diminuta*.

TABLE BXII.α.109. Characteristics of the different strains of *Phenylobacterium immobilis*^a

Characteristic	Strain (laboratory designation) ^b																						
	A ₆	A ₁₁	A ₁₂ ^c	A ₁₃	A ₁₄	C ₂ ^d	ET ^e	J ₁ ^f	J ₂	K ₂ ^g	K ₃	K ₅	L	M ₁₁	M ₁₃	M ₁₅	N ^h	R ⁱ	Z ₅	Z ₆ ^j	Z ₇	Z ₈	
Carbon source for enrichment ^k	A	A	A	A	A	C	C	C	C	C	C	C	C	P	P	P	C	C	C	C	C	C	
Growth on:																							
Chloridazon	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	
Antipyrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	
Pyrimidin	-	+	-	-	-	+	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	
Colonies ^l	R	R	S	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S	R	R	R	R	
Cells tend to clump	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	
Serological subgroup ^m	V	III	V	V	V	II (m)	V (m)	IV	IV	I	II	II	III	I (m)	III	III	IV	III	V	V	V	I/II	
Number of plasmids	3	2	2	4	2	1	3	3	4	2	2	2	4	nd	nd	3	3	3	4	6	4	3	
Plasmid size (mDa)	8.6, 10.3, 11.7, 14.0	16.3, 18.8	16.3, 32.8	10.3, 15.2, 15.7, 21.0	10.3, 15.2, 15.7, 21.0	170	8.6, 11.7, 14.0	11.7, 14.0, 19.1	10.6, 22.6, 133, 304	14.0, 21.5	14.0, 20.2	14.0, 21.5	11.7, 14.0, 17.3, 213	nd	nd	11.0, 17.0, 22.6	9.0, 17.7, 262	8.6, 11.7, 14.0	6.4, 11.7, 14.0, 19.8	7.7, 8.6, 11.7, 13.4, 14.0, 19.1	11.7, 13.4, 14.0, 19.8	11.7, 13.4, 14.0, 19.1	

^aFor symbols see standard definitions; nd, not determined.^bStrains were isolated from soils in the following locations: A₆, A₁₁, M₁₅, Z₅, Z₆, Z₇, Z₈ - Hohenheim, Germany; A₁₁, A₁₂, A₁₃, M₁₁, M₁₃ - Wimpfen, Germany; C₂ - Cardwell, Australia; ET - Ecuador; J₁, J₂ - Lyngby, Denmark; K₂, K₃, K₅ - Eldoret-Nakuru, Kenya; L - Limburgerhof, Germany; N - Lincoln, Nebraska USA; R - Rothschaig, Germany.^cATCC 35972, DSM 2115.^dCCM 3864.^eATCC 35973, DSM 1986.^fATCC 35974, DSM 2116.^gATCC 35975, DSM 2117.^hATCC 35976, DSM 2113.ⁱCCM 3865.^jATCC 35977, DSM 2114.^kA, antipyrin; C, chloridazon; P, pyrimidin.^lR, rough; S, smooth.^m(m), marginal position.

The detection of *Phenylobacterium immobile* as a new genus was the unintentional result of studies on the breakdown of the herbicide chloridazon. Chloridazon is the active ingredient of the herbicide Pyramin®. This compound has been used since about 1960 for the control of weeds in sugar beet and beet root cultures. The decomposition of chloridazon (formerly named pyrazon) was demonstrated to be a microbial process (Drescher and Otto, 1969; Frank and Switzer, 1969). The isolation of bacteria with the ability to grow on chloridazon as the sole carbon source was described by Engvild and Jensen (1969) and by Fröhner et al. (1970). Since 1969, more than 20 different strains that grow with the herbicide chloridazon or the structurally related analgesics antipyrin and pyramidon have been isolated from soils from various places over the world (Table BXII.α.109).

All attempts to identify the different isolates of chloridazon-degrading bacteria on the basis of routine characters have led to unsatisfactory results. The high nutritional specialization of these bacteria, which grow optimally on man-made compounds and utilize poorly only a few normal carbon sources, is their most distinguishing feature, along with the fact that nearly all biochemical tests give negative results.

P. immobile seems to be a typical inhabitant of the upper aerobic part of the soil. Different strains have been isolated from soil samples from various locations all over the world. All efforts to isolate chloridazon-degrading bacteria which grow at 37°C have failed. For this purpose, soil and water samples from hot springs, from near-volcanic regions, and from regions with tropical climates were subjected to chloridazon enrichment. Attempts to demonstrate the breakdown of chloridazon in soil or in mud samples under anaerobic conditions failed. In one case, a slow degradation of chloridazon in river water was observed, but efforts to isolate chloridazon-degrading bacteria from this specific water sample failed. However, one cannot rule out the possibility that phenylobacteria may occur in aquatic habitats.

The technique for the enrichment of chloridazon-degrading bacteria leads to the isolation of organisms that are able to utilize synthetic molecules not normally encountered in nature. Obviously, the chemical character of the selective agent (heterocyclic plus a phenyl moiety) is a special challenge for microbial cells, for which only a special sort of organism (*Phenylobacterium immobile*) seems to have the adequate response. This view is supported by the fact that in many different soil samples, the same sort of bacteria are always isolated. Furthermore, efforts to isolate the same bacteria with L-phenylalanine as the selective agent are unsuccessful. Enrichment techniques and nutritional characteristics raise the question of which substrates are used by these bacteria in their natural environments. Using an immunofluorescence membrane filter technique, evidence has been obtained that *Phenylobacterium immobile*, or serologically closely related organisms, also occur in soils which have never been treated with the herbicide. This suggests that these bacteria are able to survive in nature without their optimal growth substrate by utilizing other substrates—perhaps L-phenylalanine or mixtures of organic compounds such as pyruvate, succinate, malate, fumarate, and L-glutamate, which allow moderate growth.

ENRICHMENT AND ISOLATION PROCEDURES

The following procedure leads to the isolation of bacteria that are able to utilize as a sole carbon source man-made compounds not normally encountered in nature. As a synthetic substrate for selective enrichment, either the herbicide chloridazon, or the analgesics antipyrin or pyramidon (Fig. BXII.α.125) can be ap-

plied. Chloridazon, the active ingredient of the herbicide Pyramin®, can be obtained from Riedel de Haen, D-30918 Seelze, Germany; antipyrin and pyramidon (4-dimethylaminoantipyrin) are available from Sigma-Aldrich Chemie, D-89555 Steinheim, Germany. About 300 g soil or compost is mixed with 0.5 g chloridazon, antipyrin, or pyramidon, and the preparation is incubated at 30°C or room temperature in a flower pot and regularly moistened with water. Degradation of the xenobiotic compound can be observed chromatographically (TLC or HPLC) by testing the excess water that drains from the flower pot for the presence of the xenobiotic. Decomposition is complete when the xenobiotic is no longer detectable, and a new compound corresponding to the dephenylated heterocyclic moiety of the xenobiotic appears, usually after one to several weeks, depending on the soil. A 5 g sample of the active soil is then placed into an Erlenmeyer flask containing 50 ml of mineral salts medium¹ supplemented with the xenobiotic as the carbon source at a concentration of 0.04–0.1%.

This culture is incubated on a rotary shaker at 30°C, and degradation is monitored chromatographically. When the decomposition of the xenobiotic is complete, 1 ml of the culture fluid is transferred into a new Erlenmeyer flask. After 5–10 transfers, a sample of the liquid culture is streaked onto agar plates containing the same medium. Single colonies, which normally appear after 1–3 weeks, are picked and again streaked onto agar. After 5–10 transfers, pure cultures can usually be obtained. Growth on chloridazon mineral salts agar allows the removal of chloridazon to be perceived as a clearance zone around the bacterial colonies (see Fig. BXII.α.126). Since none of the *P. immobile* strains grows on ordinary complex media, the inoculation of a complex agar medium can be used for testing purity. This medium contains per liter deionized water: peptone, 5 g; meat extract, 3 g; yeast extract, 5 g; NaCl, 5 g. Growth on this medium indicates contamination of the culture.

MAINTENANCE PROCEDURES

For short-term preservation, the bacteria are transferred onto agar at intervals of 2–3 weeks. For long-term preservation, a bacterial suspension in skim milk is dropped onto silica gel grains and stored at 4°C. Good results are obtained with this method when transfer is repeated every 2–3 years; some of the strains have been viable even after a period of 10 years. Storage at –80°C of a concentrated bacterial suspension in fresh chloridazon-mineral salts medium supplemented with 15% glycerol has resulted in good viability after a storage of more than 8 years.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Nutritional specialization is a main feature of *Phenylobacterium immobile*. Therefore, tests for growth on chloridazon or antipyrin are of special importance. Optimal growth and maximum cell yield are achieved at 30°C in mineral salts medium with chloridazon or antipyrin at 0.4–1 g/l. Under these conditions, a dou-

1. The mineral salts medium has the following composition per liter deionized water: Na₂HPO₄·12H₂O, 0.7 g; KH₂PO₄, 0.3 g; (NH₄)₂HPO₄, 0.7 g; (NH₄)H₂PO₄, 0.3 g; (NH₄)₂SO₄, 0.1 g; trace element solution (see text) 1 ml; vitamin B₁₂ solution (0.03 mg/ml), 1 ml; MgSO₄·7H₂O, 0.25 g; CaCl₂·6H₂O, 0.05 g. To avoid precipitates, the magnesium and calcium salts are each dissolved separately. Trace element solution per liter deionized water: MnSO₄·4H₂O, 400 mg; ZnSO₄·7H₂O, 400 mg; FeCl₃·6H₂O, 200 mg; CuSO₄·5H₂O, 40 mg; H₃BO₃, 500 mg; (NH₄)₂MoO₄·4H₂O, 200 mg; KI, 100 mg; biotin, 100 mg. Either chloridazon, antipyrin, or pyramidon is added as the carbon source at a concentration of 0.4–1 g/l. The pH of the medium is 7.0.

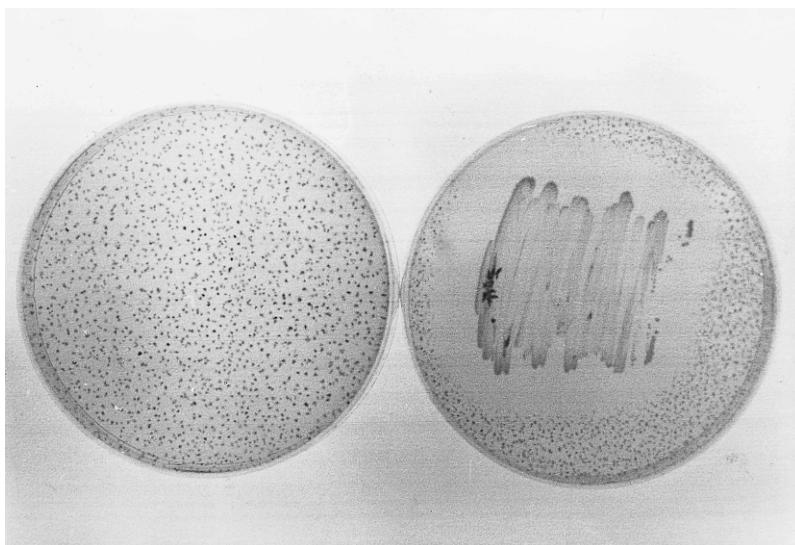


FIGURE BXII.α.126. Agar plates containing mineral salts medium with 2 g/l chloridazon. Fine crystals of chloridazon have precipitated in the agar. On the right-hand plate, a clearance zone around the bacterial smear of *Phenylobacterium immobile* has formed by chloridazon degradation. At the left margin of the bacterial smear, a large crystal is visible, which corresponds to the dephenylated heterocyclic moiety of chloridazon, the main metabolite of chloridazon degradation.

bling time of 7–8 h is observed, and, depending on the strain, a yield of about 0.4–1.0 g/l bacteria (wet weight) of culture fluid is obtained. Mineral salts medium with either 0.2% chloridazon or 0.1% antipyrin as the carbon source, supplemented with 15 g/l agar, allows good growth of *Phenylobacterium immobile*. Growth on agar may be made visible as follows: Agar plates containing mineral salts medium with 2 g/l chloridazon show a fine precipitate of chloridazon crystals in the agar. During growth on this agar, *Phenylobacterium immobile* removes the crystals by degradation, and a clearance zone around the bacterial smear develops (Fig. BXII.α.126). In agar cultures 4 or more weeks old, a new, and in most cases, relatively large sort of crystals forms within the bacterial smear. These crystals have been identified as the dephenylated heterocyclic moiety of chloridazon, which is a dead-end metabolite of chloridazon degradation.

The osmotic sensitivity and nutritional specialization of *Phenylobacterium immobile* do not allow the use of routine media for biochemical characterization. Therefore, tests have to be performed in modified media on which the bacteria are able to multiply. Mineral salts medium with 0.5 g chloridazon or 1 g antipyrin as the carbon source can be applied for testing catalase, oxidase, antibiotic susceptibility, and arginine-, lysine- and ornithine-decarboxylase. Mineral salts medium containing 1 g peptone, 1 g yeast extract, and 0.5 g antipyrin or chloridazon can be used for testing urease, indole reaction, Voges–Proskauer, methyl red, H₂S production, production of nitrite from nitrate, gelatin, starch, cellulose, and esculin hydrolysis.

DIFFERENTIATION OF THE GENUS *PHENYLOBACTERIUM* FROM OTHER GENERA

Small subunit rRNA sequence analysis has shown sequence similarities between *P. immobile* and several *Caulobacter* and *Mycoplana* species, as well as one *Afipia* genospecies. A phylogenetic tree reflecting the relationships of *P. immobile* and selected members of the *Caulobacteraceae* group is shown in Fig. BXII.α.127. The sequence similarity values suggest a close phylogenetic relation between these members of the *Alphaproteobacteria*, despite great

differences in their phenotypes and ways of life. *P. immobile* is not prosthecate like *Caulobacter*; however, it shares several properties, such as an oligotrophic nature with ready growth on dilute, complex medium (below 0.1% organic material), a strictly respiratory and aerobic metabolism, and a similar mol% G + C content of the DNA with *Caulobacter*. *Phenylobacterium* is Gram-negative like *Mycoplana* but, unlike *Mycoplana*, it does not exhibit branching filaments that fragment into motile, irregular rods—a trait that has led several workers to place *Mycoplana* in the order *Actinomycetales*. From DNA–RNA hybridization studies *M. dimorpha* and *M. bullata* seem to be remote relatives of the family *Rhizobiaceae*, a family whose members were also found to show some sequence similarity with *Phenylobacterium*; however, *Phenylobacterium* is neither a nitrogen fixer nor associated with plants. *Afipia* is the causal organism of cat scratch disease, whereas *Phenylobacterium* seems to be nonpathogenic. Comparative sequence analysis of the small subunit rRNA has demonstrated that the root-nodulating *Bradyrhizobium japonicum* and the budding bacteria *Blastobacter denitrificans* form a tight phylogenetic group with the human pathogens *Afipia felis* and *Afipia clevelandensis* (Willems and Collins, 1992).

TAXONOMIC COMMENTS

Analysis of the 16S rDNA sequence² (1466 nucleotides) of the type strain (strain E), obtained by direct sequencing of the PCR

2. The 16S rDNA of the type strain was amplified by polymerase chain reaction using primers 27f and 1522rN from the 5'- and 3'-ends of the gene. Direct sequencing of the amplified product was performed using primers to conserved regions of the rRNA. The complete sequence of the 16S rDNA of the type strain, comprising 1466 nucleotides, was deposited at the EMBL Nucleotide Sequence Database under the accession number Y18216. Potential diagnostic targets for *Phenylobacterium*-specific probes were identified by analyzing the complete small subunit rRNA sequence data set (ARB data base; approximately 13,000 sequences) using the probe design and probe match tools of the ARB program package. The most promising diagnostic probes are: 5'-CCUGAUCGCCGAGAGAU-3' (*E. coli* pos. 1000–1017) and 5'-AAUGGUACUGCCGAGGUU-3' (*E. coli* pos. 1151–1168).

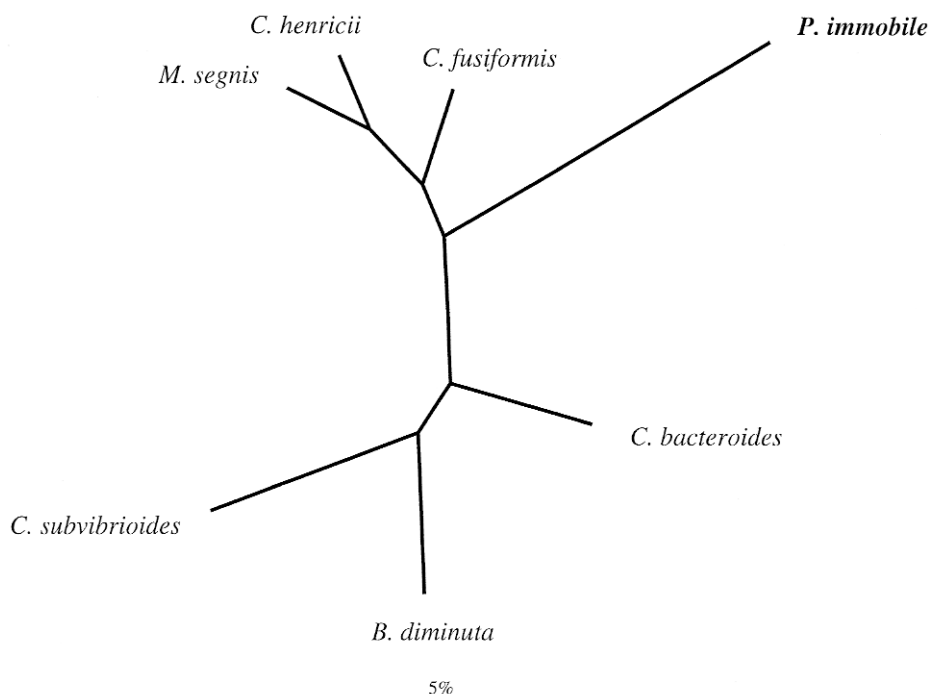


FIGURE BXII.α.127. Phylogenetic tree reflecting the relationships of *Phenylobacterium immobile* and selected members of the *Caulobacteraceae* group (*Caulobacter* spp., *Mycoplana* spp., and *Brevundimonas diminuta*). Only type strains for which 16S rRNA sequences are available are shown. The bar indicates 5% estimated sequence divergence. The tree was constructed on the basis of the complete small subunit rRNA sequence data (ARB data base, approximately 13,000 sequences. Compare: Ludwig, W. and Strunk, O. 1996 ARB: a software environment for sequence data.). The tree topology was evaluated by performing maximum parsimony and distance matrix analyses of the complete small subunit rRNA data set or a subset comprising all almost complete proteobacterial sequences together with reference data from selected representatives of the other bacterial lines of descent, respectively. Multiple data sets which varied with respect to the selection of alignment positions included were analyzed applying the alternative treeing methods. Alignment positions were included or excluded according to their degree of conservation. The majority of results support the depicted tree topology, which is based upon a data set comprising alignment positions that share identical residues in at least 50% of all sequences from members of the *Caulobacteraceae* group.

amplified gene product, reveals that *Phenylobacterium immobile* is a member of the *Caulobacteraceae*. Sequence similarities of 91.6–94.5% have been found for various *Caulobacter* species (*C. henricii*, *C. fusiformis*, *C. vibrioides*, *C. crescentus*, *C. bacteroides*, and *C. subvibrioides*). In addition, 95.9% similarity is obtained with *Afpia* genosp. 14, 94.3% with *Caulobacter segnis* (*Mycoplana segnis*), 93.2% with *M. bullata*, and 93.1% with *Brevundimonas diminuta*. Significantly, lower similarities of 90% and below are obtained with species of *Rhizobium*, *Paracoccus*, *Agrobacterium*, and other members of the *Alphaproteobacteria*. These results are in accordance with partial sequence analysis of 16S rRNA data of the type strain (Ludwig et al., 1984). Based on the formerly applied nucleotide cataloging method, *Phenylobacterium immobile* is a member of the *Alphaproteobacteria* with highest similarity (S_{AB} values of 0.51) to *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*) and *Rhizobium leguminosarum*.

Several other features support the phylogenetic position of *Phenylobacterium* that has been established by small subunit rRNA sequence analysis. The presence of 2,3-diamino-2,3-dideoxy-D-glucose as a lipid A constituent of *Phenylobacterium immobile* indicates its relationship to the *Alphaproteobacteria*. This unusual sugar has also been detected in *Rhodopseudomonas viridis*, *R. palustris*, *R. sulfovoridis*, *Brevundimonas diminuta*, *B. vesicularis*, and

Nitrobacter winogradskyi (Weckesser and Mayer, 1987). Busse and Auling (1988) have shown that polyamines may serve as a useful chemotaxonomic marker within the *Proteobacteria*. Like other species of the *Alphaproteobacteria*, *P. immobile* contains *syn*-homospermidine exclusively, as well as ubiquinone Q-10, composed of 10 isoprenoid units (R.M. Kroppenstedt, J. Eberspächer, and F. Lingens, unpublished). Weak serological reactions of *Phenylobacterium immobile* occur only with members of the *Alphaproteobacteria* (Dorfer et al., 1985) and not with any other Gram-negative bacteria.

DNA hybridization tests reveal 100% similarity of DNA preparations among four different strains with DNA of strain R. No similarity has been found using DNA from *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, or calf thymus. Morphological, physiological, biochemical, and nutritional studies, and serological and enzymological data have also demonstrated the high degree of similarity among the more than 20 different isolates. Therefore, the isolates have been grouped together in one single species. On the other hand, minor, but significant, differences exist in cell and colony morphology, in plasmid pattern, in the utilization of the xenobiotics chloridazon, antipyrin and pyrimidon, and in serological properties that allow a classification into 5 serological subgroups (Table BXII.α.109).

The construction of *Phenylobacterium*-specific probes using 16S rDNA sequence analysis data should allow the detection of *Phenylobacterium immobile* and closely related bacteria in environmental samples without prior enrichment with xenobiotics. This technique using diagnostic target probes seems to be more specific than the previously applied serological method. Based on the probe examination of environmental samples, it would seem to be feasible to isolate *P. immobile* or *P. immobile*-like organisms without using the selective enrichment technique for growth on the xenobiotics chloridazon, antipyrin, or pyrimidon.

List of species of the genus *Phenylobacterium*

1. ***Phenylobacterium immobile*** Lingens, Blecher, Blecher, Blobel, Eberspächer, Fröhner, Görisch, Görisch and Layh 1985, 38^{VP}
im.mo' bi.le. L. adj. *immobile* nonmotile.

The characteristics are as described for the genus and in Tables BXII.α.109 and BXII.α.110. *P. immobile* uses relatively few simple carbon sources. Growth occurs on L-glutamate, pyruvate, fumarate, succinate, and malate, but more poorly than on chloridazon or L-phenylalanine. A mixture of these compounds, e.g., pyruvate plus L-glutamate, yields better growth than do single compounds, although the growth rate and cell yield are still lower than with chloridazon. No growth occurs on more than 40 compounds tested as possible carbon sources, including various sugars,

alcohols, carboxylic acids, and amino acids. A similar spectrum of compounds is negative for acid or gas production. Complex media with 10–20 g/l peptone or meat extract plus yeast extract does not support growth, whereas with 0.5–2 g/l peptone plus yeast extract, growth occurs, but more slowly than on chloridazon. *Phenylobacterium immobile* is osmotically sensitive; the addition of 5–7 g NaCl per liter chloridazon mineral salts medium result in considerable growth inhibition, 10 g NaCl leads to total growth inhibition.

The mol% G + C of the DNA is: 65–66.5 (Bd); strain C₂ has a somewhat higher value of 68.5 (Bd).

Type strain: E, ATCC 35973, DSMZ 1986.

GenBank accession number (16S rRNA): Y18216.

TABLE BXII.α.110. General characteristics of *Phenylobacterium immobile*^a

Characteristic	<i>Phenylobacterium immobile</i>
Cell diameter, µm	0.7–1.0
Cell length, µm	1.0–2.0
Cells single, in pairs or short chains	+
Motility	–
Spores, sheaths, prosthecae	–
Pigmented	–
Long rods or chains or elliptical cells in old cultures	d
Gram-stain, Ziehl-Neelsen-stain, capsule-stain	–
Colonies smooth	d
Colonies rough	d
Strictly aerobic	+
Catalase	+
Oxidase	w
Growth at 4°C and 37°C	–
Optimal growth temperature, °C	28–30
Growth at pH 4 and 9	–
Optimal pH	6.8–7.0
Growth inhibition by 0.5–0.7% NaCl	+
Growth factor vitamin B ₁₂	+
Optimal growth on chloridazon, antipyrin, and phenylalanine (after long lag phase)	+
Fluorescent pigment on phenylalanine medium	+

(continued)

ACKNOWLEDGMENTS

The author is greatly indebted to Dr. Wolfgang Ludwig, Institute of Microbiology, Technical University of Munich, for processing 16S rDNA data and constructing a phylogenetic tree, and to Prof. Dr. Michael Schlömann and his coworkers from the Institute of Microbiology, University of Stuttgart, for his introduction into the 16S-rDNA sequencing method.

FURTHER READING

Lingens, F., R. Blecher, H. Blecher, F. Blobel, J. Eberspächer, C. Fröhner, H. Görisch, H. Görisch and G. Layh. 1985. *Phenylobacterium immobile* gen. nov., sp. nov., a Gram-negative bacterium that degrades the herbicide chloridazon. Int. J. Syst. Bacteriol. 35: 26–39.

TABLE BXII.α.110. (cont.)

Characteristic	<i>Phenylobacterium immobile</i>
Non-fluorescent pigment on chloridazon medium	+
Moderate growth on glutamate, succinate, pyruvate, fumarate, malate	+
Growth on sugars, alcohols, amino acids (40 compounds tested)	–
Growth on aromatic compounds (18 compounds tested)	–
Acid or gas from sugars and alcohols (34 compounds tested)	–
Growth on complex media	–
Growth on dilute complex media (0.5–2 g/l organic material)	+
NH ₄ ⁺ and NO ₃ [–] used as N-sources	+
Denitrification	–
N ₂ -fixation	–
Gelatine, casein, starch, esculin hydrolyzed	–
Litmus milk	–
H ₂ S from cysteine or thiosulfate	w
Methyl red, Voges-Proskauer, indole	–
Mol% G + C of DNA (Bd)	65–68.5

^aFor symbols see standard definitions.

Order VI. **Rhizobiales** *ord. nov.*

L. DAVID KUYKENDALL

Rhi.zo.bi.a'les. M.L. n. *Rhizobium* type genus of the family *Rhizobiaceae*; *-ales* ending to denote an order; M.L. fem. n. *Rhizobiales* the *Rhizobium* order.

The order *Rhizobiales* is a phenotypically heterogeneous assemblage of Gram-negative bacteria and is based solely on 16S rRNA gene sequence analysis.

Type genus: **Rhizobium** Frank 1889, 338.

Families of the Order Rhizobiales

- I. Family *Rhizobiaceae*
- II. Family *Bartonellaceae*
- III. Family *Brucellaceae*
- IV. Family *Phyllobacteriaceae*
- V. Family *Methylocystaceae*
- VI. Family *Beijerinckiaceae*
- VII. Family *Bradyrhizobiaceae*
- VIII. Family *Hyphomicrobiaceae*
- IX. Family *Methylobacteriaceae*
- X. Family *Rhodobiaceae*

Family I. **Rhizobiaceae** Conn 1938, 321^{AL}

L. DAVID KUYKENDALL

Rhi.zo.bi.a'ce.ae. M.L. n. *Rhizobium* type genus of the family; *-aceae* ending to denote a family; M.L. fem. n. *Rhizobiaceae* the *Rhizobium* family.

The family *Rhizobiaceae* is a phenotypically heterogeneous assemblage of aerobic, Gram-negative rod-shaped bacteria and is based solely on 16S rRNA gene sequence analysis. (Table BXII.α.111)

Type genus: **Rhizobium** Frank 1889, 338.

Genera of the Family Rhizobiaceae

- I. Genus *Rhizobium*
- II. Genus *Agrobacterium*
- III. Genus *Carbophilus*
- IV. Genus *Chelatobacter*
- V. Genus *Ensifer*
- VI. Genus *Sinorhizobium*

TABLE BXII.α.111. Some determinative phenotypic features of the genera of the family *Rhizobiaceae*^a

Characteristic	<i>Rhizobium</i> , <i>Sinorhizobium</i> , <i>Allorhizobium</i>	<i>Agrobacterium</i>	<i>Carbophilus</i>	<i>Chelatobacter</i>	<i>Ensifer</i>
Reproduce by budding at one pole of the cell	—	—	nd	d	+
Attach endwise to various host bacteria and may cause lysis of the host cells	—	nd	—	—	+
Some strains induce hypertrophisms in plants as root nodules with or without symbiotic nitrogen fixation	+ ^b	— ^b	nd	nd	nd
Some strains induce tumorous galls in plants	— ^b	+ ^b	nd	nd	nd
Aminopolycarboxylic acid, nitrilotriacetic acid can be utilized as a sole source of carbon/energy and nitrogen	—	—	nd	+	nd
Can grow chemolithoautotrophically with carbon monoxide (CO) as a sole carbon and energy source	—	—	+	nd	nd

^aFor symbols see standard definitions; nd, not determined.

^bTraditionally, *Agrobacterium* has been associated with the incitement of tumorous galls and/or hairy roots in plants, whereas *Rhizobium*, *Sinorhizobium*, and *Allorhizobium* have been associated with the symbiotic induction of root nodules. However, because these features are plasmid-mediated, this is not a reliable basis for classification of these genera.

Genus I. Rhizobium Frank 1889, 338^{AL}

L. DAVID KUYKENDALL, JOHN M. YOUNG, ESPERANZA MARTÍNEZ-ROMERO, ALLEN KERR AND HIROYUKI SAWADA

Rhi.zo'bi.um. Gr. n. *rhiza* a root; Gr. n. *bios* life; M.L. neut. n. *Rhizobium* that which lives in a root.

Rods 0.5–1.0 × 1.2–3.0 μm. Nonsporeforming. Gram negative. **Motile by 1–6 peritrichous flagella.** Fimbriae have been described on some strains. **Aerobic**, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. Optimal temperature for growth, 25–30°C; some species can grow at temperatures >40°C. Optimal pH for growth, 6–7; range pH 4–10. Generation times of *Rhizobium* strains are 1.5–5.0 h. **Colonies** are usually white or beige, circular, convex, semi-translucent or opaque, raised and mucilaginous, **usually 2–4 mm in diameter within 3–5 days on yeast-mannitol-mineral salts agar (YMA).** **Growth on carbohydrate media is usually accompanied by copious amounts of extracellular polysaccharide.** Pronounced turbidity develops after 2 or 3 days in aerated or agitated broth. Chemoorganotrophic, utilizing a wide range of carbohydrates and salts of organic acids as sole carbon sources, without gas formation. Cellulose and starch are not utilized. **Produce an acidic reaction in mineral-salts medium containing mannitol or other carbohydrates.** Ammonium salts, nitrate, nitrite, and most amino acids can serve as nitrogen sources. Strains of some species will grow in a simple mineral salts medium with vitamin-free casein hydrolysate as the sole source of both carbon and nitrogen, but strains of many species require one or more growth factors such as biotin, pantothenate, or nicotinic acid. Peptone is poorly utilized. Casein, starch, chitin, and agar are not hydrolyzed. **All known *Rhizobium* species include strains which induce hypertrophisms in plants as root nodules with or without symbiotic nitrogen fixation.** Some cells of symbiotic bacterial species enter root hair cells of leguminous plants (Family Leguminosae) via invagination or by wounds (“crack entry”) and elicit the production of root nodules wherein the bacteria engage as intracellular symbionts, usually fixing nitrogen. Many well-defined nodulation (*nod*) and nitrogen fixation (*nif*) genes are clustered on large plasmids or megaplasmids (pSyms). Plasmid transfer between species results in the expression and stable inheritance of the particular plant-interactive properties of the plasmid-donor species. Plant host specificity is usually for a few legume genera but may, in some strains, include a wide variety of legume genera and is to some extent determined by the chemical structure of the lipochito-oligosaccharide *Nod* factors produced. These chitin-like molecules induce nodule organogenesis in the absence of bacteria. **In root nodules the bacteria occur as endophytes that exhibit pleomorphic forms, termed “bacteroids”, which reduce or fix gaseous atmospheric nitrogen into a combined form utilizable by the host plant.**

The mol% G + C of the DNA is: 57–66.

Type species: *Rhizobium leguminosarum* (Frank 1879) Frank 1889, 338 (*Schinzia leguminosarum* Frank 1879, 397.)

FURTHER DESCRIPTIVE INFORMATION

Introductory Note This treatment of the genus *Rhizobium* also includes information on the closely related genera *Agrobacterium*, and *Sinorhizobium*. The rationale for this treatment is provided under Taxonomic Comments.

Following Bradbury (1986), strains in past literature called *Agrobacterium* biovar 1 are herein referred to as *Agrobacterium tumefaciens*. *Agrobacterium* biovar 2 strains are referred to as *Agrobacterium rhizogenes*. *Agrobacterium* biovar 3 strains are referred to

as *Agrobacterium vitis* (See Table BXII.α.114 in the chapter on the genus *Agrobacterium*). Where necessary, the ability of pathogenic strains to cause crown gall tumors or the hairy root condition, previously attributed to strains using the names “*Agrobacterium tumefaciens*” and “*Agrobacterium rhizogenes*”, is indicated by reference to the tumorigenic or rhizogenic ability, respectively, of strains in species of *Agrobacterium*. Nonpathogenic strains previously named *Agrobacterium radiobacter* are referred to either as nonpathogenic strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or merely as nonpathogenic *Agrobacterium* if the species designation has not been identified. Ti or Ri plasmids determine the pathogenic status of strains (see below). Species comprising pathogenic or nonpathogenic strains can be reported as tumorigenic as a (Ti strain) or (Ti), as rhizogenic as a (Ri strain) or (Ri), or as nonpathogenic strains of the species where relevant.

Morphology The formation of star- or rosette-shaped aggregates of cells by several *Agrobacterium* strains has been described by Beijerinck and van Delden (1902), Stapp and Knösel (1956) and Knösel (1962). Granules of poly-β-hydroxybutyrate are common in older cells, so that upon simple staining the rods appear banded. Strains of *R. leguminosarum* often contain metachromatic granules, demonstrated by staining with methylene blue, washing with dilute iodine, and staining with neutral red (Graham and Parker, 1964). Fig. BXII.α.128 shows a cell of *R. leguminosarum* biovar trifolii.

All species are motile by one to six flagella. For most species examined, insertion is peritrichous. Strains in species with a single flagellum (*R. galegae*, *R. mongolense*, *Sinorhizobium fredii*, *Sinorhizobium saheli*, *Sinorhizobium teranga*, and *Sinorhizobium xinjiangense*) also appear to have peritrichous organization expressed as polar or sub-polar insertion. Only *R. hainanense* is reported as being unambiguously polarly flagellated.

Cell wall composition The cell wall structure of *Rhizobium* is generally similar to that of other Gram-negative bacteria. The peptidoglycan consists of glutamic acid, alanine, diaminopimelic acid, and amino sugars. In addition, leucine, phenylalanine, serine, and aspartic acid have been detected in relatively large amounts in the peptidoglycan layer of several pathogenic strains. Lipopolysaccharide (LPS) cell wall composition varies from strain to strain but consistently contains 2-keto-3-deoxyoctanoic acid (KDO), uronic acids, glucosamine, glucose, mannose, rhamnose, fucose, and galactose (Carlson, 1982). The structures of LPS from a number of species have been determined, and all of them contain the unusually long 27-hydroxyoctacosanoic acid (Jeyaretnam et al., 2002; Sharypova et al., 2003). *Rhizobia* also have an unusually complex composition of membrane phospholipids, among them phosphatidylcholine, and under conditions of phosphorus limitation, phospholipids can be replaced by membrane lipids that do not contain phosphorus (López-Lara et al., 2003).

Fine structure As revealed by electron microscopy and biochemical analyses, cellulose-containing fibrils are formed by pathogenic *Agrobacterium* strains during their attachment to plant cells *in vitro*. These fibrils anchor the bacteria to the plant cell surface

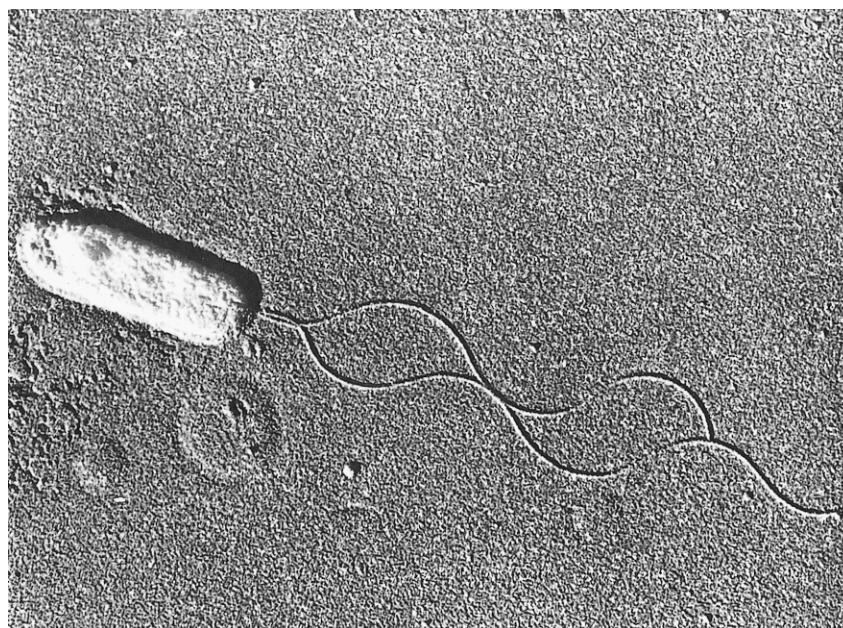


FIGURE BXII.α.128. Cell of *Rhizobium leguminosarum* biovar *trifolii* showing two polar flagella ($\times 14,000$).

(Matthysse et al., 1981). Lipopolysaccharides of the outer membrane of cell envelopes play a role in the attachment of the bacteria to the wound site of the plant (Whatley et al., 1976). Smit et al. (1989) purified an adhesin that appears to mediate the first step in attachment of nodulating *Rhizobium* and pathogenic *Agrobacterium* bacterial cells to plant root hair tips.

Colonial and cultural characteristics On carbohydrate-containing solid media, the majority of *Rhizobium* strains produce circular, low convex to convex, mucous, glistening, opaque, white to beige-colored colonies, with an entire edge and a diameter of 2–4 mm after 5–6 days of incubation at 28°C.

Most strains grow rapidly on a mineral salts medium containing yeast extract and any one of a wide variety of carbohydrates (Vincent et al., 1979). Acid is usually produced to a moderate degree from carbohydrates (Norris, 1965).

Growth on carbohydrate-containing solid media may be opaque, clear or translucent (Fig. BXII.α.129) but may also exhibit small opaque areas within a clear slime. Variants that produce small colonies often fix little nitrogen symbiotically. Growth of *Agrobacterium* on nutrient agar is moderate, whereas abundant growth is obtained on media containing yeast extract and a suitable carbohydrate such as glucose, sucrose, or lactose (see Maintenance Procedures).

Almost all strains of *Rhizobium* species—but not *Agrobacterium* species—form only white colonies on yeast extract-mannitol-mineral-salts medium containing 0.0025% Congo red.

Some strains are encapsulated. All produce abundant water-soluble extracellular polysaccharide, the principal constituent of which is acidic heteropolysaccharide (80–90%). The remaining constituents, in most strains, are neutral, unbranched, β -2-linked glucans, of which some cyclics are important for nodule development (York et al., 1980; Breedveld and Miller, 1994). Certain chromosomal genes termed *chv* or *ndv* are responsible for the production of cyclic glucans essential for either virulence or nodule development in all species of *Agrobacterium* or *Rhizobium*. All strains within a species produce the same acidic heteropolysac-

charide except for *R. leguminosarum* biovar *phaseoli*, which possesses a unique heteropolysaccharide. Curdlan production has been reported in several isolates of *R. leguminosarum* biovar *trifolii* (Ghai et al., 1981). The formation of water-insoluble β -1,3-glucans has been reported in some strains of pathogenic *Agrobacterium* (Nakanishi et al., 1976). The synthesis of glycogen by *Agrobacterium tumefaciens* strain B6 is regulated at the level of ADP-glucose synthesis (Eidels et al., 1970).

Nutrition and growth conditions The temperature range for growth, which is highly strain dependent, is 4–40°C; however, growth at 4°C is rare, and only certain species can grow at 40°C. The temperature maximum for *R. leguminosarum* is 38°C. All of the strains grow between 20°C and 28°C. *Agrobacterium rhizogenes* cannot grow above 30°C, whereas *Sinorhizobium saheli* and *Sinorhizobium teranga* can grow at 44°C (de Lajudie et al., 1994). The pH range for growth for the entire genus *Rhizobium* is 4–10.

Intermediates of the tricarboxylic acid cycle and several amino acids can be utilized as sole sources of carbon. The majority of *Agrobacterium tumefaciens* strains can grow on a minimal medium with nitrate or ammonium salts as the nitrogen source. *Agrobacterium rhizogenes* strains do not utilize nitrate unless biotin is supplied; some strains require both L-glutamic acid and biotin. Strains belonging to *Agrobacterium rubi* require L-glutamic acid and yeast extract (Starr, 1946; Lippincott and Lippincott, 1969; Keane et al., 1970).

Metabolism and metabolic pathways The principal mechanisms of glucose catabolism in *Rhizobium* are the Entner-Doudoroff pathway and the pentose cycle (Katznelson and Zagallo, 1957; Vardanis and Hochster, 1961; Martínez-De Drets and Arias, 1972; Arthur et al., 1973, 1975; Ronson and Primrose, 1979). It is unlikely that the Embden-Meyerhof-Parnas pathway operates in *Rhizobium* spp. because activities of fructose-1,6 diphosphate aldolase and 6-phosphofructokinase are low. Polyols are substrates for an inducible dehydrogenase that converts mannitol to fructose and arabitol to xylulose (Martínez-De Drets and Arias,

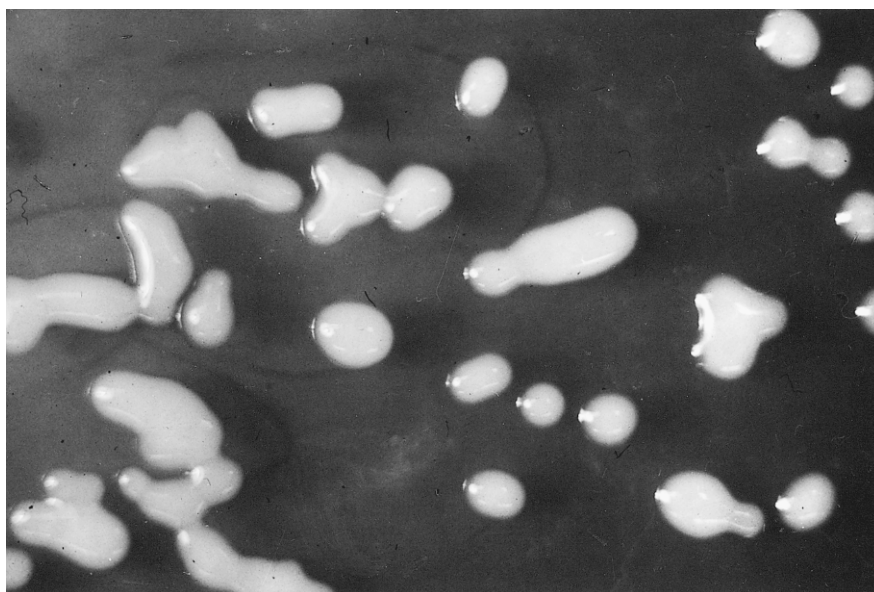


FIGURE BXII.α.129. Colonies of *Rhizobium leguminosarum* on mineral-salts mannitol agar ($\times 1.5$).

1970). L-Arabinose is metabolized to α -ketoglutarate (Duncan, 1979).

The tricarboxylic acid cycle is operative, and the enzymes of the glyoxylate bypass are present (Johnson et al., 1966; Arthur et al., 1973; Chern et al., 1976a). Pyruvate carboxylase is an important anaplerotic enzyme (Chern et al., 1976b; Ronson and Primrose, 1979).

Glucuronic acid and glucaric acid are metabolized via 2-keto-3-deoxy-D-glucaric acid to α -ketoglutaric acid (Chang and Feingold, 1970). The initial step in the catabolism of L-sorbose by some *Agrobacterium tumefaciens* strains is the reduction to sorbitol, followed by oxidation of the latter compound to D-fructose (Van Keer et al., 1976). The majority of *Agrobacterium* strains characteristically oxidize a large number of carbohydrates (disaccharides, bionic acid, and several monosaccharides) to the corresponding 3-uloses (Bernaerts and De Ley, 1960a, b; Fukui et al., 1963; De Ley et al., 1966). The vigorous and unusual oxidation of lactose to 3-ketolactose is, so far, unique to *Agrobacterium tumefaciens* and is the basis of a simple and specific diagnostic test for the rapid differentiation of this species from other *Agrobacterium* and *Rhizobium* spp. that have been tested (Bernaerts and De Ley 1963; de Lajudie et al., 1994). These specific oxidations are catalyzed by an inducible hexopyranoside:cytochrome *c* oxidoreductase (D-glucoside 3-dehydrogenase), containing flavin adenine dinucleotide as cofactor (Hayano and Fukui, 1967; Van Beeumen and De Ley, 1968; Nakamura and Tyler, 1977). Although an "alpha-3"-ketoglucosidase was detected in a strain of *Rhizobium* (Hayano and Fukui, 1970; Hayano et al., 1973), 3-ketosucrose and 3-ketolactose are probably not involved as essential intermediates in the metabolism of sucrose and lactose, respectively (Kurowski and Pirt, 1971). Conditions have been worked out for increasing the yield of 3-ketoglycosides (Tyler and Nakamura, 1971; Fensom et al., 1974; Kurowski et al., 1975).

Anaerobic growth by nitrate reduction has been reported for *Sinorhizobium fredii* (Hynes et al., 1985) and *Sinorhizobium meliloti* (Daniel et al., 1982). *Sinorhizobium fredii* produces N_2O and *Sinorhizobium meliloti* produces N_2 as the end products of denitrification.

Rhizobium contains at least two soluble cytochromes *c*: a cytochrome c_{552} and a cytochrome c_{556} . Cytochrome c_{552} has been sequenced (Van Beeumen et al., 1980). It belongs to the cytochrome *c* sequence class IB (*sensu* Ambler, 1973) and, of all known procaryotic cytochromes *c*, shows the highest amino acid sequence homology with mitochondrial cytochrome *c* of tuna fish (Van Beeumen et al., 1980). Cytochrome c_{556} from *Rhizobium* belongs to the cytochrome *c* sequence class II (*sensu* Ambler, 1973), because its single heme group is bound near the C-terminus (Van Beeumen et al., 1980).

Auxotrophic mutants of *Rhizobium* are sometimes symbiotically defective and the isolation and study of such mutants has provided insights into the biochemical prerequisites of symbiosis (Kuykendall, 1981). For example, auxotrophy toward adenine, uracil, and leucine is often associated with symbiotic ineffectiveness in *Sinorhizobium meliloti* (Dénarié et al., 1976).

Fatty acids Fatty acid profiles have been studied for some species. Using principal components analysis of whole cell fatty acid methyl esters (FAME), Jarvis et al. (1996) reported three clusters: 1) *R. leguminosarum*, *R. etli*, *R. tropici*, *Agrobacterium rhizogenes*, *Sinorhizobium fredii*, and *Sinorhizobium meliloti*; 2) *R. galegae*, *Agrobacterium tumefaciens*, *Agrobacterium rubi*, and *Agrobacterium vitis*; and 3) *R. huakuii* and *R. loti*, (now allocated to *Mesorhizobium*). Sawada et al. (1992d) could differentiate *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium vitis* using fatty acid profiles. *Agrobacterium tumefaciens* could be separated from *A. vitis* based on quantitative differences in $C_{16:1}$ and $C_{18:1}$ acids, and the presence of $C_{17:0}$ cyclo acids. Quantitative differences in $C_{19:0}$ cyclo, and the presence of $C_{15:0}$ iso 3OH acids and the absence of $C_{18:1}$ 3OH acids differentiated *Agrobacterium rhizogenes* from the other two species (Sawada et al., 1992d). Tighe et al. (2000) studied 600 strains and found that the fatty acid composition of all the fast-growing species differed from that of *Bradyrhizobium* and *Mesorhizobium*, but there were no clear differences among fast-growing genera except for the relative concentration of $C_{16:0}$ 3OH fatty acid, which was quantitatively lower in *Sinorhizobium* than in most *Rhizobium* strains. Species-level identification was,

however, possible in many cases, and thus the data of Tighe et al. (2000) is a valuable resource.

Genetics A circular linkage map of the *R. leguminosarum* chromosome was first constructed by Beringer and Hopwood (1976). The genome of *Sinorhizobium meliloti* has also been mapped (Kondorosi et al., 1977; Meade and Singer, 1977). These two species have similar chromosomal gene arrangements (Beringer et al., 1987). *Sinorhizobium meliloti* has a chromosome size of 3.7×10^6 bp, and its sequence has been reported (Galibert et al., 2001).

There are only incomplete comparisons of *Rhizobium* spp. by DNA–DNA reassociation. DNA–DNA reassociation data are available for the following species: *R. galegae*, *R. leguminosarum*, and *R. tropici* (Martínez-Romero et al., 1991); *Sinorhizobium fredii* and *Sinorhizobium meliloti* (de Lajudie et al., 1994); and *Sinorhizobium saheli* and *Sinorhizobium teranga* (de Lajudie et al. 1994). An early study was made of *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium rubi* (De Ley 1972, 1974).

Easily amplified DNA regions called amplicons, apparently controlling both adaptability and biological interactions, have been documented in *Rhizobium* genomes (Palacios et al., 1998). As these become better defined through mapping and DNA sequencing, their structure can be expected to be of particular interest.

Plasmids Plasmids and megaplasmids as large as 1600 kb can constitute as much as 50% of the total genome. Sequences of plasmids have been reported (Freiberg et al., 1997; Barnett et al., 2001; Finan et al., 2001).

Hypertrophic activity and plasmids All species (though not all strains) of *Rhizobium* and *Agrobacterium* are associated with plant hypertrophisms and, with a few exceptions, are associated with root hypertrophisms. Hypertrophy can involve formation of either nitrogen-fixing nodules in *Rhizobium* or of oncogenous (tumorous) galls or hairy roots in *Agrobacterium*. These activities are confined to particular species. Genes (pSym, pTi, and pRi) associated with hypertrophisms are usually carried on large plasmids. These genes and the associated hypertrophying activity play a central role in the ecology of these genera. Plant transformations using the agrobacterial tumorigenic system are now routine (see reviews by Kado, 1991; Zambryski, 1992; Hooykaas and Beijersbergen, 1994). Transformations in filamentous fungi have also been reported (De Groot et al., 1998).

Plasmid-regulated nitrogen fixation Some members of the *Rhizobiaceae* and *Phyllobacteriaceae* are characterized by their ability to incorporate functional genetic elements as plasmids or symbiotic islands (Sullivan and Ronson 1998) which permit them to establish pathogenic (oncogenic) or symbiotic nitrogen-fixing relationships with plants. Nitrogen-fixing symbioses involving members of the *Rhizobiaceae* are restricted to plants of the family Leguminosae (van Rhijn and Vanderleyden, 1995), with one exception. *Rhizobium* cells contain as many as 10 naturally occurring plasmids ranging in size from less than 100 kb to megaplasmids of more than 1000 kb. In some instances, the combined length of plasmids and megaplasmids approximates that of the chromosome, meaning that up to 50% of the *Rhizobium* genome is not in the chromosome. Both *nod* and *nif* genes controlling nodulating and nitrogen-fixing ability have always been found clustered together on one or more *Rhizobium* plasmids or megaplasmids called pSym. Numerous genetics studies have delineated *Rhizobium* genes essential for legume nodulation and sym-

biotic nitrogen fixation (Rossen et al., 1984; Török et al., 1984; Egelhoff and Long, 1985; Egelhoff et al., 1985; Jacobs et al., 1985; Debellé et al., 1986; Evans and Downie, 1986; Göttfert et al., 1986; Horvath et al., 1986; Rostas et al., 1986; Shearman et al., 1986; Aguilar et al., 1987; Fisher et al., 1987; Honma and Ausubel, 1987; Cremers et al., 1988; Davis et al., 1988; Surin and Downie, 1988; Cervantes et al., 1989; De Maagd et al., 1989; Schwedock and Long 1989, 1994; Barnett and Long, 1990; Economou et al., 1990; Honma et al., 1990; Surin et al., 1990; Baev et al., 1991, 1992; Kondorosi et al., 1991a, b; Rushing et al., 1991; Baev and Kondorosi, 1992). The nodulation-controlling genes are organized into several coordinately regulated operons. For example, the common *nodABC* operon is present in all legume symbiotic strains and can complement strains in different genera. For *R. etli*, *nodA* is separated from *nodBC* (Vázquez et al., 1991; Vázquez et al., 1993), and for *Mesorhizobium loti*, *nodB* is independent of *nodAC* (Scott et al., 1996). Others may be present in different species as allelic variants, such as *nodEF*, and these are host-specific and hence not interchangeable. Such *nod* or *hsn* genes are sometimes present only in certain strains.

pSyms are not essential for survival of the *Rhizobium* strains in soil. Non-nodulating soil bacteria identified as *Rhizobium* species have been isolated which can only form nodules after transconjugation with related symbiotic strains. Transfer of symbiotic plasmids among *Rhizobium* species has been reported under laboratory conditions and has been demonstrated by sequence comparison of natural isolates. Martínez et al. (1987) demonstrated nitrogen-fixing nodules formed by *Agrobacterium tumefaciens* carrying a conjugally transferred pSym from *R. tropici* (as *R. phaseoli* type II). Similar results were obtained for transconjugants of *Agrobacterium tumefaciens* containing pSym from *Rhizobium* strains that nodulate *Phaseolus vulgaris* (Brom et al., 1988). A pSym of *R. leguminosarum* biovar trifolii has been introduced into 15 non-nodulating bacterial isolates identified as *M. loti*, *R. leguminosarum*, *R. tropici*, *Sinorhizobium meliloti*, and four isolates related to *R. leguminosarum* (Sivakumaran et al., 1997). By comparison of *nifH* sequence type to 16S rRNA gene sequence type, Haukka et al. (1998) demonstrated that similar symbiotic genes could be found in different 16S rRNA gene backgrounds, indicating horizontal transfer across species boundaries.

There is considerable variation in the nodulating and nitrogen-fixing capacity of individual strains. In *Rhizobium* strains with the capacity for symbiotic activity, most if not all genes that specify and regulate nodulating and nitrogen-fixing abilities are carried on one or more plasmids. In this respect, members of the genus differ from *Bradyrhizobium* (see the chapter on the genus *Bradyrhizobium*), in which all symbiosis-controlling genes have been shown to be carried on the chromosome, and *Mesorhizobium* (see the chapter on the genus *Mesorhizobium*), in which many strains have symbiosis-controlling genes located on the chromosome. The systematics of these plasmid-determined symbiotic associations is reviewed in Young and Johnston (1989).

The expression of nodulation genes is controlled by the presence of flavonoids excreted by the host plant. For, example, the regulatory *nodD* gene controls *nodABC* expression. Flavonoids produced by various legumes seem to interact specifically with particular NodD proteins, which vary in structure according to *Rhizobium* species, as do the Nod Factors (NF). Compatibility between flavonoid and NodD protein is thought to be a major factor in host specificity. (For detailed reviews of nodulation and nitrogen fixation genetics and biochemistry, see Schultze et al., 1994; van Rhijn and Vanderleyden, 1995; Dénarié et al., 1996.)

Another regulation gene, *nolR*, has been reported to be common in symbiotic species of *Rhizobium* and *Sinorhizobium*. This gene was not found in species of *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, or *Agrobacterium* (Kiss et al., 1998). This gene repressed the expression of both *nodABCII* and *nodD* genes, resulting in decreased Nod factor production. Recently, a novel family of *nod* gene inducers, aldonic acids, was reported for *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Bradyrhizobium* (as *B. lupini*) strains (Gagnon and Ibrahim 1998).

Root nodules induced by some strains of symbiotic *Rhizobium* contain substances called rhizopines (Murphy et al., 1987, 1995), analogous to the opines found in pathogenic *Agrobacterium* strains (see later discussion). Those described so far are substituted scyllo-inosamines. Both synthetic and catabolic genes are located on pSym. Genes involved in synthesis are active in the symbiotic bacteroid state and catabolic genes are active in the free-living cells (Murphy et al., 1988; Dessaux et al., 1998). The likely ecological benefit of rhizopine activity for producing strains has been reported (Gordon et al., 1996). Rhizopine production has been reported for *R. leguminosarum* biovar *viciae* and *Sinorhizobium meliloti*. No rhizopine production was detected from isolates of *R. etli*, *R. tropici*, or *R. leguminosarum* biovar *trifolii* and biovar *phaseoli* (Wexler et al., 1995). Rhizopine synthesis (*mos*) and catabolism (*moc*) genes from *R. leguminosarum* and *Sinorhizobium meliloti* have been sequenced (Wexler et al., 1996b). Further study showed that some non-symbiotic soil bacteria, including *Arthrobacter*, *Aeromonas*, *Alcaligenes*, and *Pseudomonas*, could catabolize rhizopine. No DNA sequences homologous to *nodB* and *nodC* and no effective nodulation on *Medicago sativa* were detected in two strains that were related to *Sinorhizobium meliloti* based on partial 16S rDNA sequence analysis (Gardener and Bruijn, 1998); this result indicated that the gene(s) related to rhizopine metabolism may be carried on the chromosome or on plasmids other than pSym.

Two megaplasmids in *Sinorhizobium meliloti* are involved in the effective nodulation of alfalfa (Hynes et al., 1986). In *R. leguminosarum* (Hynes and McGregor, 1990), as in *R. etli* CFN42, plasmids other than the pSym (*nod-nif* plasmid) are required for an effective symbiosis and may carry *lps* genes (García-de los Santos and Brom, 1997; Vinuesa et al., 1999).

Plant specificity in nitrogen fixation The legume-nodulating ability of *Rhizobium* species appears to be specific to a few plant species or genera. An exception is *Sinorhizobium* sp. strain NGR234, which nodulates 112 legume genera (Pueppke and Broughton, 1999). In such instances, nodulation can occur without nitrogen fixation. Since Lerouge et al. (1990) established the chemical structure of the nodule-inducing compound produced by *Sinorhizobium meliloti*, a general hypothesis has been proposed: host specificity in all legume microsymbionts is related to the chemical structure of specific lipochitooligosaccharide Nod factors (Dénarié et al., 1992, 1996). Nodulation factors of strain NGR234 include variants of Nod factors (Price et al., 1992). Structures of Nod factors produced by several species have been described (Poupot et al., 1993, 1995; Lorquin et al., 1997; Yang et al., 1999; Snoeck et al., 2001; Pacios-Bras et al., 2002).

Plasmid-mediated plant-pathogenic (oncogenic) activity in *Agrobacterium* spp. The early literature on plasmid-mediated plant pathogenic (oncogenic) activity was reviewed by Nester et al. (1984). A recent review is given by Binns and Costantino (1998). Oncogenic (tumorigenic or rhizogenic [hairy root]) activity in the four plant-pathogenic *Agrobacterium* species is mediated by genes that are largely or wholly borne on one or more

large (>150 kb) plasmids. Tumorigenic activity is conferred by Ti plasmids and rhizogenic activity is conferred by Ri plasmids. Tumorigenic genes on the Ti plasmid comprise (a) T-DNA genes, and (b) virulence (*vir*) genes. Wounding of susceptible plant tissue activates *vir* genes that facilitate the transfer of a component of the Ti (or Ri) plasmid, the T-DNA (8–22 kb). The T-DNA fragment is integrated into the plant nucleus apparently at random (Chyi et al., 1986), in one or more copies (Chilton et al., 1980; Lemmers et al., 1980; Willmitzer et al., 1980; Zambryski et al., 1980). T-DNA carries all necessary genes for tumor growth, the most important for tumorigenesis being those associated with auxin and cytokinin synthesis, which are expressed in the plant.

Comparative analysis indicates a correlation between sequence structure of the 16S–23S rRNA intergenic spacer region and the type of Ti plasmid (nopaline, vitopine, or octopine/cucumopine) present in strains of *Agrobacterium vitis* (Ottens et al., 1996).

Another large Ri plasmid is involved in the hairy root disease of plants caused by rhizogenic *Agrobacterium* strains (Moore et al., 1979; White and Nester, 1980a). Little overall sequence similarity to other Ti plasmids has been detected (White and Nester, 1980b). There is one small region of conserved similarity between the Ri plasmid and an octopine Ti plasmid (pTi-B6806), but the former shows no similarity to the T-DNA region of the latter plasmid. The Ri plasmid is compatible with other Ti plasmids and thus represents a new incompatibility class of plasmids (White and Nester, 1980b).

Large plasmids that are not involved in pathogenic activity have been found in nonpathogenic strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Merlo and Nester, 1977; Sheikholeslam et al., 1979). In addition, large plasmids have been discovered in addition to the Ti plasmid in several tumorigenic *Agrobacterium* strains. Some strains possess an additional 2.1×10^6 base-pair linear chromosome in addition to two or more very large plasmids (Allardet-Servent et al., 1993). The sequence of the *A. tumefaciens* C58 genome was reported (Goodner et al., 2001).

Opines are unusual amino acid derivatives produced in tumor or hairy root tissues induced by pathogenic strains of *Agrobacterium* species. Opines are synthesized from common plant compounds but are not utilized by plants or by most microorganisms. They are utilized by *Agrobacterium* strains that carry the specific opine-inducing plasmids associated with oncogenicity. The opine concept as originally proposed was that specific genes associated with T-DNA permitted the synthesis of opines from plant photosynthetic products in a form whose availability as nutrients was restricted to tumorigenic strains bearing the relevant plasmid (Schell et al., 1979). At least eleven opines have been identified: octopine, lysopine, nopaline, succinamopine, leucinopine, cucumopine, heliopine, chrysopine, mikimopine, agropine, and agrocinopines (Chang et al., 1989; Dessaux et al., 1992; Chilton et al., 1995). In addition, imino acids (Moore et al., 1997), manopine (Petit et al., 1983), and vitopine (Szegedi et al., 1988) have been reported. It is unlikely that this list is exhaustive (Moore et al., 1997). Opine catabolism by pathogenic *Agrobacterium* strains is mediated by genes, located on one or more plasmids including the Ti plasmid, that are not transferred to the plant cell nucleus (Montoya et al., 1977). Pathogenic strains can utilize more than one opine (Moore et al., 1997). Some opines can also induce conjugal transfer of the Ti plasmid to nontumorigenic strains and may contribute to the dissemination of the infectious plasmid (Gelvin 1992; Guyon et al., 1993). Analysis of

the distribution of Ti plasmids in terms of their opine genes showed that the ecology of plasmid-bearing strains is highly complex (Moore et al., 1997). In only a few samples could field outbreaks of crown gall be traced to a clonal origin of infection. In most collections, field tumors were induced by Ti plasmids of more than one opine type. Field tumors of some hosts yielded no detectable opines, even though opine-utilizing bacteria were present. Bacterial isolates from other hosts (plum and cherry) showed the best correspondence between the opine in tumors (nopaline) and the presence of bacteria that catabolized that opine. However, several unusual opine catabolic combinations were identified, including isolates that catabolized a variety of opines but were nonpathogenic. There are indications of some specificity between pathogenic *Agrobacterium* species, opine type, and host plant (Lopez et al., 1988; Sawada et al., 1992a). The opine concept (Schell et al., 1979) assumed specific utilization of opines by tumorigenic *Agrobacterium* strains bearing relevant plasmids, and that these compounds could not be utilized by other soil organisms. Since the original proposal, however, it has become clear that a wide range of soil organisms have the capacity to metabolize opines. These include *Pseudomonas* species (Beaulieu et al., 1983; Tremblay et al., 1987b) and Gram-positive coryneform bacteria (Tremblay et al., 1987a). These data collectively suggest that mechanisms explaining the involvement of opines may be more complex than the original model.

At present, oncogenic activity is associated with *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, and *Agrobacterium vitis*. There may be several additional pathogenic bacterial populations that also merit classification as species. Bouzar et al. (1995) records a pathogen associated with aerial infections of *Ficus benjamina*, and Sawada and Ieki (1992b) report phenotypically distinct strains isolated from affected plants.

Pathogenic host range and pathogenicity The host range of tumorigenic *Agrobacterium* strains is reported to be very wide. De Cleene and De Ley (1976) described at least 640 plant species belonging to 331 genera in 93 families of dicotyledon and gymnosperm plants as susceptible to transformation by *Agrobacterium* (Ti strains) (such as *Agrobacterium tumefaciens*). De Cleene and De Ley (1981) reported 37 plant species belonging to 30 genera in 15 families of dicotyledonous plants as susceptible to transformation by *Agrobacterium* (Ri strains) (such as *Agrobacterium rhizogenes*). None of the 250 monocotyledonous species investigated was susceptible to the disease, except some members of the orders Liliales and Arales. Bradbury (1986) listed almost 400 plant species affected by tumorigenic strains and over 50 plant species affected by rhizogenic strains.

The inference that crown gall- and hairy-root-inducing strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have wide host ranges is based on reports of the many hosts from which the pathogens have been isolated and on the many reports of tumor induction in experimental inoculations. However, there have also been indications of host specificity within some populations (Panagopoulos and Psallidas, 1973; Anderson and Moore 1979; Moore and Cooksey 1981; Paulus et al., 1991a, b; Palumbo et al., 1998). In some instances, strains are pathogenic to a relatively narrow range of host plants (Unger et al., 1985). Furthermore, although crown gall has been reported on some host species in some countries, it is not necessarily found in these same hosts in other countries where crown gall is known. The family of Ti plasmids may have restricted host ranges in varying degrees and chromosomal background may affect specificity. It

seems clear that some strains naturally infect host plants from several unrelated genera whereas others are more specific (Paulus et al., 1991b). The nature of specificity, whether it is a function of the bacterial strain or of the plasmid (Loper and Kado 1979; Close et al., 1985; D'Souza et al., 1993), has not been generally confirmed, although modifications to the Ti-plasmid have been implicated (Paulus et al., 1991a). There is no indication of host range specificity as it occurs in the pathogenic species and pathovars of *Pseudomonas* or *Xanthomonas*. Although *Agrobacterium rubi* is identified as a species isolated from galls on the canes of *Rubus* spp., the specificity of this pathogen to *Rubus* is in doubt because it has been shown to have a wide host range (Sawada et al., 1992a). *Agrobacterium vitis* is found as the predominant tumorigenic species specific to *Vitis* spp. (Thies et al., 1991), and *Agrobacterium vitis* strains have occasionally been isolated from other hosts, such as *Actinidia* (Sawada and Ieki 1992a). *Agrobacterium vitis* appears to be unique among pathogenic *Agrobacterium* species in being associated with a root decay symptom (Burr et al., 1987).

Upon infection of wounded plant tissues, tumorigenic *Agrobacterium* strains can transform plant cells into autonomously proliferating cells. In nature, the swellings mostly occur at the transition zone between the stem and the root system of the host plant, hence the name "crown gall disease." Small spherical growths or elongated ridges can occur on the stems of *Rubus* spp. such as raspberry and bramble bushes. Some oncogenic strains cause hairy root on susceptible plants (such as apple trees and roses) but cause crown gall on other plants. On some plants (e.g., *Kalanchoë*), oncogenic strains of *Agrobacterium* spp. can induce the formation of teratomata, characterized by the development of aberrant shoots, leaves, or roots developing from the tumor tissue. The type of disease produced (differentiated or undifferentiated tumors) is probably determined by both the bacterial Ti plasmid and the host plant (Gresshoff et al., 1979).

A prerequisite for tumorigenesis is the wounding of the host. Infection can occur during various stages of the life of a plant via wounds caused by growth, germination (e.g., peaches and almond), subterranean insects, or mechanical injuries (e.g., pruning, grafting, and replanting of trees in nurseries).

Tumorigenic and rhizogenic activity was initiated by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* at 20°C and 27°C, respectively. The latter temperature, however, was not conducive to tumorigenesis by *Agrobacterium vitis*. Temperature effects were mediated by the choice of host plant (Charest and Dijon, 1985).

Crown gall disease seldom kills plants, but growth is often impaired and stunted. Significant damage and economic loss can occur on stone fruit and grape (De Cleene, 1979).

Plasmid exchange between *Rhizobium* and *Agrobacterium* species Intergeneric transmissibility of Ti and nodulating plasmids has been demonstrated from nodulating *Rhizobium* spp. to tumorigenic *Agrobacterium* spp. (Martínez et al., 1987; Brom et al., 1988; Abe et al., 1998), and from tumorigenic *Agrobacterium* spp. to nodulating *Rhizobium* spp. (Hooykaas et al., 1977) and to *Phyllobacterium myrsinacearum* (van Veen et al., 1988); this supports a close relationship for these genera and points to promiscuous plasmid exchange between taxa. Novikova and Safronova (1992) reported transconjugants of *Agrobacterium tumefaciens* harboring the pSym genes of *R. galegae* that formed an effective symbiosis with *Medicago sativa*. The finding by Nesme et al. (1987) that crown gall that occurred in a poplar nursery was caused by naturally occurring resident mixed populations of both *Agrobacte-*

rium tumefaciens (Ti strains) and *Agrobacterium rhizogenes* (Ti strains) supports the idea that Ti plasmids may be promiscuous in the resident tumorigenic *Agrobacterium* species. Plasmid homologies do not correlate with any numerical classification of pathogenic *Agrobacterium* spp. (Currier and Nester, 1976), hence it is generally assumed that plasmid-borne Ti and Ri genes are readily transmitted within and between strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

Although plasmids conferring pathogenicity and nodulation are transmissible between pathogenic and nodulating genera in the laboratory, in nature these characteristics appear to be specific to the particular species. The relative specificity between particular nodulating *Rhizobium* spp. and their legume symbionts, and the pathogenic specificity indicated for *Agrobacterium vitis* and, perhaps, *Agrobacterium rubi* suggests that some plasmid incompatibilities exist, causing a restriction on transmission or subsequent gene expression. Furthermore, Bouzar et al. (1993) and Otten et al. (1996) demonstrated a correlation between the form of resident plasmids and host chromosome, suggesting possible restrictions on exchange in nature.

Agrocin and trifolitoxin activity Agrocin 84 from the non-pathogenic *Agrobacterium rhizogenes* strain 84 (ICMP 3379; NCPPB 2407) (New and Kerr, 1972; Kerr and Htay, 1974) is plasmid-encoded (Ellis et al., 1979). It is a toxic analog of an adenine nucleotide (Roberts et al., 1977) and selectively inhibits pathogenic *Agrobacterium* strains harboring a nopaline plasmid. It is effective against *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* but not *Agrobacterium vitis* or *Agrobacterium rubi* (Ma et al., 1985; van Zyl et al., 1986; Psallidas 1988; Sawada et al., 1992a). Sensitivity towards agrocin 84 is determined by the Ti plasmid. Dipping seeds, roots, or wounded plant surfaces in suspensions of strain 84 has been used with success worldwide for the biological control of crown gall disease (Moore and Warren, 1979; Kerr, 1980). Strain 84 is available in commercial preparations as a biological control agent and has found wide application. *Agrobacterium vitis* strains are insensitive to agrocin 84. Other agrocin-producing strains, effective against *Agrobacterium vitis*, have been isolated (Staphorst et al., 1985; Chen and Xiang 1986; Webster et al., 1986; Webster and Thompson 1988; Xie et al., 1993).

Trifolitoxin (TFX) is a post-translationally modified peptide antibiotic produced by *R. leguminosarum* biovar trifolii T24 (Breil et al., 1993). TFX is toxic to non-producing strains within a distinct taxonomic group of the *Alphaproteobacteria*, and it appears to give an ecological advantage for nodulation by the producing strain. Eight genes have been identified for the production of this toxin (Triplett et al., 1994; Breil et al., 1996).

Bacteriophages Lysogeny for either active plaque-forming or defective bacteriophages is widespread in tumorigenic *Agrobacterium* spp. Morphological, biological, and physicochemical properties and genetic relationships of several of the isolated phages or phage-like particles have been determined (Beardsley, 1955; Zimmerer et al., 1966; Stonier et al., 1967; De Ley et al., 1972; Manasse et al., 1972; Vervliet et al., 1975). Virulent bacteriophages of *Rhizobium* were the subject of numerous studies published prior to 1950 (Allen and Allen, 1950), but *Agrobacterium* phages, although not as extensively studied in earlier times, have more recently been isolated from sewage and soil (Roslycky et al., 1963; Boyd et al., 1970a, b).

The range of hosts susceptible to a particular *Rhizobium* bacteriophage is highly variable. In some instances, it is limited to relatively few strains within a single host species; in others, it may

cross taxonomic boundaries. Cross-infection studies of *Sinorhizobium meliloti* and *Sinorhizobium fredii* (Hashem et al., 1996) indicate that bacteriophage lysis is not usually sufficiently specific to identify species or individual strains. Some strains of *Rhizobium* are lysogenic. Bacteriocins have been reported (Roslycky, 1967; Venter et al., 2001), as well as a parasitic *Bdellovibrio*.

Antigenic structure Early serological studies indicated that strains belonging to *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* could be distinguished from each other by serological reactions (Keane et al., 1970; Lopez, 1978). Alarcón et al. (1987) and Sawada et al. (1992c) found serological heterogeneity between these species and with *Agrobacterium vitis*. *Rhizobium* spp. show extensive cross-reaction with *Agrobacterium* strains (Graham, 1971).

Most serological reactions (agglutination, gel diffusion, precipitation, and fluorescent antibody) show strain specificity and have traditionally been of great value in identifying particular strains of *Rhizobium* in nodules of field plants or in laboratory investigations. The agglutination reaction, using crushed nodule extracts, is the most widely used for field work because of its simplicity, although it is complicated by cross-reactions and autoagglutination. Surface antigens, although useful for strain recognition, are limited in their usefulness for species identification. Early work, such as that of Vincent and Humphrey (1970), who reported on the antigen structure in the "biovars" of *R. leguminosarum*, should now be reinterpreted in the light of modern taxonomic revisions of the species. Sawada et al. (1992b) differentiated several serogroups in *Agrobacterium vitis* using a slide agglutination test.

Ecology *Rhizobium* occurs worldwide in soils and especially in the rhizosphere of plants. As many as 10^6 – 10^7 cells/g soil of symbiotic *Rhizobium* have been reported. *Rhizobium* strains capable of degrading 2-sulfonato-fatty-acid-methyl-esters (Masuda et al., 1995) and *Agrobacterium* strains capable of utilizing phthalate (Nomura et al., 1989) were reported as common soil inhabitants in contaminated soils. The identification of these strains as authentic rhizobia and agrobacteria needs confirmation.

Natural interactions between *Rhizobium* strains within the rhizosphere are complex, as indicated by the extent to which different *Rhizobium* populations are shown to compete in the infection processes. Agrocin-producing strains have a proven role in competing with tumorigenic *Agrobacterium* strains to inhibit infection. A similar competitive process occurs when attempts are made to nodulate seedling legumes with effective *Rhizobium* strains. Naturally occurring strains that are nonefficient in nitrogen fixation can be more effective in infecting and nodulating plants, thereby limiting plant growth (Triplett and Sadowsky, 1992). Strains of *Rhizobium* that are non-nodulating and occurring naturally in soils have been well documented (Soberón-Chávez and Nájera, 1989; Segovia et al., 1991). Symbiotic *Rhizobium leguminosarum* biovar trifolii strains have been reported as natural endophytes in the roots of rice (Yanni et al., 1997), and *R. etli* strains, as endophytes of maize (Gutiérrez-Zamora and Martínez-Romero, 2001). Plant-pathogenic *Agrobacterium* species have also been isolated from a crown gall tumor on alfalfa (Palumbo et al., 1998).

Although bacteria within the genus *Rhizobium* have been shown to have a worldwide distribution, unique species may be isolated from limited geographic regions, normally related to the distribution of their hosts (Martínez-Romero and Caballero-Melgado, 1996). *Rhizobium* species may have been spread interna-

tionally with inoculated legume plants, with seed (Pérez-Ramírez et al., 1998), or soil. Caballero-Mellado and Martínez-Romero (1999) reported that soil fertilization limited the genetic diversity of *Rhizobium* in bean nodules. Vance (1998) reviewed agronomic aspects of commercial inoculants that are used to enhance legume crop cultivation.

Agrobacterium strains have also been reported in a variety of human clinical specimens (CDC group Vd-3) (Lautrop, 1967; Riley and Weaver, 1977; Gilardi, 1978a; Rubin et al., 1980). They are usually 3-ketolactose-positive and nonpathogenic to tomato. It is believed that these clinical isolates occur either as incidental inhabitants in the patient or as contaminants introduced during sample manipulation. The authenticity of these strains as true *Agrobacterium* spp. needs to be confirmed.

Antibiotic sensitivity *Rhizobium* strains are resistant to a variable spectrum of antibiotics (Davis, 1962). Most are susceptible to tetracycline. Although there is wide strain-to-strain variation in resistance, *Rhizobium* strains are intrinsically more sensitive than *Bradyrhizobium* to tetracycline, penicillin G, viomycin, vancomycin, and streptomycin. Streptomycin-resistant mutants, which are usually effective as nodulating strains, are important in ecological field studies on strain competition. In general, wild-type *Agrobacterium* tumorigenic species have been reported to be sensitive to chlorotetracycline, gentamicin, neomycin, novobiocin, oxytetracycline, and tetracycline (Kerstens et al., 1973) but are commonly resistant to nalidixic acid. Growth is inhibited by low concentrations (3–780 µg/ml of medium) of metacycline, doxycycline, sigmamylin (tetracycline + oleandomycin), and triacetyloleandomycin (Goedert, 1973).

ENRICHMENT AND ISOLATION PROCEDURES

Although *Rhizobium* strains are common soil inhabitants, they are best isolated from freshly excised legume root nodules. Identification is relatively easy if strains are isolated from host plant nodules. Isolation is difficult if strains are isolated directly from the soil or if strains are non-infective, unless they have unique genetic markers. Isolation of symbiotic species from soil generally requires the use of trap hosts, which are leguminous plants grown in the soil and from which nodules are selected for subsequent isolation of rhizobia. Nodules collected in the field can be temporarily stored in small vials containing silica gel held under a cotton plug.

In order to isolate symbionts, healthy root nodules—with a small portion of root attached if they are very small (<1.0 mm)—are surface sterilized by exposure to commercial 3% H₂O₂ solution or 5% commercial hypochlorite (3% available chlorine) solution for 5–60 min depending on their size. This treatment is followed by a wash in sterile water. The nodules are crushed in a small drop of sterile 0.05% peptone or 0.1M phosphate and a loopful of the suspension is streaked onto surface-dried plates of yeast extract-mannitol agar (YMA)¹ prepared without CaCO₃. Alternatively, a small loopful of the crushed nodule suspension can be streaked onto successive plates of agar medium. Large nodules can be sliced with a sterile scalpel blade and portions of the interior removed with a needle. Bacteria can readily be isolated from young galls and nodules on different parts of plants.

1. Yeast extract-mannitol agar (YMA) contains (g/l of distilled water): D-mannitol, 10.0; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.1; CaCO₃, 4.0; yeast extract (Difco), 0.4; agar, 15.0; pH 6.8–7.0. Sterilize at 121°C for 15 or 30 min depending on the volume. The CaCO₃ is omitted for the preparation of pour plates or for liquid medium.

Isolation from older hypertrophying tissue is more difficult. Incubation is at 28°C for 3 or more days. Well-isolated, white, mucoid, glistening, hemispheric colonies are restreaked onto fresh plates for subsequent confirmation, which requires reinfection of the original host under careful aseptic conditions where uninoculated controls are devoid of nodules. If heavy fungal contamination is expected, the agar medium used for initial isolation should contain 0.002% actidione. *Rhizobium* strains grow poorly on 0.04% peptone/1% glucose mineral salts agar and show little pH change. This medium can serve as a useful contamination check: colony formation in 2 days at 28°C and a marked pH change are not characteristic of *Rhizobium*.

MAINTENANCE PROCEDURES

YMA slant cultures in sealed containers can be stored for 2–3 years or longer at 2°C. Cultures usually survive for around 2 months at 15°C. Long-term storage at –20°C or –80°C is recommended: turbid suspensions from fresh broth cultures are mixed with equal volumes of sterile 80% glycerol in water and allowed to stand for 1 hr at room temperature before storing in small aliquots in the freezer. Individual aliquots are thawed as required. Norris (1963) described a preservation method using small porcelain beads which, after inoculation and drying over silica gel, can be used individually to inoculate YMB for subsequent recovery of the bacteria.

Stock cultures of pathogenic species may be routinely maintained on agar slants in screw-capped vials at 4°C for 2 months on YMA or on either of the following media (in g/l of tap water): (a) glucose, 20; yeast extract, 10; CaCO₃, 20; and agar, 20; or (b) glucose, 10; yeast extract, 10; (NH₄)₂SO₄, 1.0; KH₂PO₄, 0.25; and agar, 20.

Lyophilized cultures stored at 4°C remain viable for at least 25 years.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Carbohydrate and organic acid utilization have been determined using inoculated plates of the medium of Elkan and Kwik (1968). On the dried surface of these plates are placed absorbent paper discs previously saturated with a 10% solution of the organic compound and slowly dried. During incubation at 28°C the plates are examined daily using indirect lighting against a black, non-reflecting background.

API Biotype galleries (BioMérieux, La Balme-les-Grottes, France) and similar standardized systems to test for the utilization of standard ranges of substrates are increasingly used to obtain reproducible biochemical data.

Details on the methods used to assess nodulation response under greenhouse or growth room conditions are given by Vincent (1970), and Somasegaran and Hoben (1994) have produced an excellent methods book for the novice researcher of symbiotic species. Moore et al. (1988) have provided useful advice for performing inoculation tests for pathogenicity studies and summarize recipes for diagnostic media and tests.

Sawada et al. (1995) have reported a method for detecting the presence of Ti and Ri plasmids by specific amplification of components using the polymerase chain reaction.

DIFFERENTIATION OF THE GENUS *RHIZOBIUM* FROM OTHER GENERA

Members of *Rhizobium* are distinguished from those in the related genera *Mesorhizobium* and *Phyllobacterium* by differences in growth rate, fatty acid profiles, and 16S rDNA sequence. Members of

Rhizobium are not distinguished from those in the related genera *Allorhizobium* or *Sinorhizobium* by any phenotypic characters except those that form the individual species circumscriptions. The genus *Agrobacterium* is distinguished from the genera containing nitrogen-fixing species, including *Rhizobium*, only because its members have oncogenic capabilities. *Rhizobium* is distinguished from *Sinorhizobium* not only based on differences in their 16S rDNA sequences but also on the basis of other gene sequences (Gaunt et al., 2001) and by *nolR* gene hybridization (Toledo et al., 2003).

The features that differentiate nodulating strains of *Rhizobium* species from morphologically and physiologically similar organisms are given in Tables BXII.α.112 and BXII.α.113. Strains of nonpathogenic agrobacteria and non-nodulating rhizobia can be isolated from soils and are difficult to allocate to genera or species based on characteristics reported in Table BXII.α.112. It is necessary to resort to specific molecular probes for reliable identification.

TAXONOMIC COMMENTS

Overviews of the relationships of bacterial nitrogen-fixing genera are given in Young (1992, 1994), Martínez-Romero (1994), Lindström et al. (1995, 1998), Martínez-Romero and Caballero-Mellado (1996), and Young and Haukka (1996).

At that time of publication of the first edition of *Bergey's Manual of Systematic Bacteriology*, the family *Rhizobiaceae* comprised *Rhizobium* (Jordan, 1984a), *Bradyrhizobium* (Jordan, 1984a), *Phyllobacterium* (Knösel, 1984a), and *Agrobacterium* (Kerstens and De Ley, 1984a).

Since then, the relationships of nodulating, nitrogen-fixing species have been investigated by comparative analysis of 16S rDNA sequence data. Sequences of the type strains, obtained from international databases, have been subjected to various forms of algorithmic and parsimonious analysis in order to establish their phylogenetic relationships (Sawada et al., 1993; Willems and Collins 1993; de Lajudie et al., 1994, 1998b, 1998a; Nour et al., 1995; Rome et al., 1996; Young and Haukka 1996; Amarger et al., 1997; Tan et al., 1997; Lindström et al., 1998). Comparison of these analyses with others that have been published (Rome et al., 1996; Jarvis et al., 1997; de Lajudie et al., 1998a, b; van Berkum et al., 1998; Wang et al., 1998), and with Fig. BXII.α.130 of this chapter, shows how much results can vary depending on the selection of sequences and the form of analysis. Notwithstanding, all data clearly support the separation of *Bradyrhizobium* Jordan 1982 (now assigned to the family *Bradyrhizobiaceae*, Kuykendall, this volume) and *Azorhizobium* Dreyfus et al., 1988 (now assigned to the family *Hyphomicrobiaceae*, Kuykendall, this volume) as distantly related to all the other pathogenic and nodulating species. These latter species are allocated to the closely related families, *Rhizobiaceae* and *Phyllobacteriaceae*. Nitrogen-fixing and oncogenic (hypertrophying) species in *Agrobacterium*, *Rhizobium*, and *Sinorhizobium* are assigned to the *Rhizobiaceae* and species of *Mesorhizobium* (Jarvis et al., 1997; Chen and Kuykendall, this volume) are assigned to the *Phyllobacteriaceae* (Chen and Kuykendall, this volume).

The family *Rhizobiaceae* includes symbiotic and pathogenic species in the genera *Agrobacterium*, *Rhizobium*, and *Sinorhizobium*. Species allocated to these genera are found in two or three clusters. One cluster comprises *Sinorhizobium*: *Sinorhizobium fredii* (the type species), *Sinorhizobium arboris*, *Sinorhizobium kostense*, *Sinorhizobium medicae*, *Sinorhizobium meliloti*, *Sinorhizobium saheli*, *Sinorhizobium teranga*, and *Sinorhizobium xinjiangense*. *S. arboris* and *S.*

kostense were only recently described (Nick et al., 1999). The second cluster is more heterogeneous and may be considered to be represented by two subgroups. Subgroup 2a includes *R. leguminosarum* (the type species), *R. etli*, *R. gallicum*, *R. giardinii*, *R. hainanense*, *R. mongolense*, *R. tropici*, and *Agrobacterium rhizogenes*. Subgroup 2b includes *R. galegae*, *R. huautlense*, *Agrobacterium tumefaciens* (the type species), *Agrobacterium rubi*, *Agrobacterium vitis*, and *Allorhizobium undicola* (the type species). All these species have base differences amounting to less than 7% of the total 16S rDNA sequence. The extent of statistical support for individual branches and their relative positions depend on the form of phylogenetic analysis and the selection of sequences. Eardly et al. (1996), Martínez-Romero and Caballero-Mellado (1996), and Young and Haukka (1996) note anomalies in sequence analyses that are attributable to recombination events between species, a conclusion supported but qualified by Wernegreen and Riley (1999). As yet the significance and implications of recombination on the inference of phylogenetic relationships are unclear.

The family also contains, as outliers to the rhizobial species, strains named *Blastobacter* spp. and "*Liberibacter*" spp., which do not have symbiotic or pathogenic characteristics. Other strains of *Blastobacter* spp. are to be found in the families *Methylobacteriaceae* (four strains), *Bradyrhizobiaceae* (one strain), and *Sphingomonadaceae* (one strain). *Blastobacter aggregatus* ATCC 43293 and *Blastobacter capsulatus* ATCC 43294 in the *Rhizobiaceae* are therefore perhaps incorrectly named. The new genus "*Liberibacter*" represents strains of the fastidious organism that is the pathogen of citrus greening disease. This organism appears to be relatively distantly related to *Rhizobium*.

When first proposed, *Sinorhizobium* (Chen et al., 1988b) was based on only a small number of nutritional and biochemical tests, and its validity was questioned by Jarvis et al. (1992) on the basis of partial 16S rDNA sequence analysis and on the interpretation of numerical data. This genus has since been examined in greater detail and an emended circumscription of the genus has been produced (de Lajudie et al., 1994). However, this circumscription does not delineate a taxon distinct from *Rhizobium*, and the polyphasic data reported (PAGE of total proteins and carbon source utilization tests) do not support a coherent taxon. Moreover, the protein data show *Sinorhizobium fredii* (the type strain) as an outlier to the other species, and carbon source utilization data show *Sinorhizobium* species intermingled with *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium*. Support for this genus, distinct from *Rhizobium*, is based on comparative 16S rDNA sequence data alone (de Lajudie et al., 1994).

Allorhizobium, is a monospecific genus established because—as indicated by its name—comparative analysis of 16S rDNA sequence data for *Allorhizobium undicola* indicated that this organism belonged to an outlying branch (de Lajudie et al., 1998b). The closest neighboring species in their analysis was *Agrobacterium vitis*. The species *Allorhizobium undicola* is well defined based on DNA–DNA reassociation, PAGE of total proteins, and carbon source utilization tests. As with *Sinorhizobium*, the circumscription of the genus does not delineate a taxon distinct from *Rhizobium*, and the polyphasic data do not support a close relationship between *Allorhizobium undicola* and *Agrobacterium vitis* and other agrobacteria. In proposing a new genus, rather than either nominating the species as a nitrogen-fixing member of *Agrobacterium ex tempore* or allocating it to *Rhizobium ex tempore*, the authors are committed to a nomenclature in which either *Agrobacterium vitis* is renamed *Allorhizobium vitis* or is recognized in its own monospecific genus. While monospecific genera can sensibly be named

TABLE BXII.α.112. Characteristics of *Rhizobium*, *Agrobacterium*, *Allorhizobium*, and *Sinorhizobium* species^{a,b}

Characteristics	<i>Rhizobium leguminosarum</i>	<i>Rhizobium elii</i>	<i>Rhizobium galegae</i>	<i>Rhizobium gallium</i>	<i>Rhizobium giardinii</i>	<i>Rhizobium hainanense</i>	<i>Rhizobium huautlense</i>	<i>Rhizobium mongolense</i>	<i>Rhizobium tropici</i>	<i>Agrobacterium tumefaciens</i>	<i>Agrobacterium rhizogenes</i>	<i>Agrobacterium rubi</i>	<i>Agrobacterium vitis</i>	<i>Allorhizobium undicola</i>	<i>Sinorhizobium fredii</i>	<i>Sinorhizobium medicae</i>	<i>Sinorhizobium meliloti</i>	<i>Sinorhizobium saheli</i>	<i>Sinorhizobium teranga</i>	<i>Sinorhizobium xinyi</i>
Polar flagella	2-6	1-2	1-2			1		1	yes	1-4	1-4	1-4	1-4	yes	1-3		2-6	1	1	1
Pertirichous flagella or one subpolar flagellum	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-
3-ketolactose produced	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth factors required:																				
Biotin	d	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	d	-	-	-
Pantothenate	+	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Thiamine	d	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
pH range	4-9	+	5-9.5 ^c	>4 to <8	4-8.5	5-10	5-9	4-10	4-10	+	5-9	+	+	+	5-10.5	5-10	4.5-9.5	+	+	5-10.5
Grows at 28°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grows at 35°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grows at 40°C	-	-	d ^c	-	d	+	+	+	+	-	-	-	-	-	-	-	d	+	+	+
Grows in 1% NaCl	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
Grows in 2% NaCl	-	-	-	-	-	+	+	-	-	+	-	-	+	+	d	+	+	+	+	(+)
Growth in Luria-Bertani medium	-	-	-	-	-	+	+	+	+	many ^d	many ^d	few ^d	few ^d	-	-	-	+	-	-	-
Oncogenicity to few or many plant genera ^d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Symbiotic	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
nodulating/nitrogen-fixing ability ^d																				

^aFor symbols see standard definitions.

^bData are from Graham and Parker (1964), Jordan (1984a), Kersters and De Ley (1984a), Kerr (1992) and original descriptions of species.

^cUnpublished data of E.T. Wang (personal communication) using the methods of Wang et al. (1998).

^dBecause oncogenicity and nitrogen-fixing symbioses are plasmid-mediated and the stability of resulting host specificity is also uncertain, these characteristics are not reliable bases for classification or identification of these species.

TABLE BXII.α.113. Carbon source utilization tests which differentiate *Rhizobium*, *Agrobacterium*, *Allorhizobium*, and *Sinorhizobium* species^{a,b}

Substrate ^{c,d}	<i>Rhizobium</i> <i>leguminosarum</i>	<i>Rhizobium</i> <i>galgae</i>	<i>Rhizobium</i> <i>tropicum</i>	<i>Agrobacterium</i> <i>tumefaciens</i>	<i>Agrobacterium</i> <i>rhizogenes</i>	<i>Agrobacterium</i> <i>vitis</i>	<i>Allorhizobium</i> <i>undicola</i>	<i>Sinorhizobium</i> <i>fredii</i>	<i>Sinorhizobium</i> <i>meliloti</i>	<i>Sinorhizobium</i> <i>saheli</i>	<i>Sinorhizobium</i> <i>terangae</i>
Number of strains ^e	37	2	3	7	3	2	6	2	3	4	20
Acetate		+	d	+	-	+	d	+	d	+	+
N-acetylglucosamine		+	+	+	-	+	-	+	+	+	+
Aconitate		-	+	+	-	-	-	d	-	+	d
Adonitol		+	+	+	+	+	-	+	+	+	+
L-(alpha)-Alanine	+	+	+	+	d	+	+	-	+	+	d
beta-Alanine		-	-	-	-	-	d	-	+	d	-
DL-3-Amino butyrate		-	d	d	-	+	d	+	+	-	+
DL-4-Amino butyrate		d	-	-	d	-	d	-	d	d	d
DL-5-Aminovalerate		-	+	-	+	d	-	-	-	+	+
Amygdalin		-	+	+	+	d	-	+	+	+	d
D-Arabinose		+	+	+	+	d	-	+	+	+	+
L-Arabitol		-	+	+	+	-	-	+	+	-	-
Arbutin		+	+	-	+	+	+	+	+	+	+
L-Arginine	+	d	+	-	+	-	d	d	+	+	d
L-Aspartate		-	+	+	+	+	d	-	+	+	d
Butyrate		-	-	-	-	+	-	-	-	-	d
Citrate		d	d	-	+	+	-	d	-	+	-
L-Citrulline		-	d	-	+	-	-	-	-	-	-
L-Cysteine	+	-	-	-	d	-	-	-	-	-	-
Dulcitol	+	-	-	+	d	-	-	-	+	-	+
Erythritol	+	-	d	-	+	-	-	d	+	+	d
Ethanolamine		d	-	-	+	-	-	+	+	-	+
D-Fucose		+	d	+	+	+	d	d	d	-	d
Gluconate		+	+	+	+	-	-	+	-	+	+
L-Glutamate	+	-	+	+	d	d	d	-	+	+	-
Glutarate		-	d	d	-	+	-	+	-	+	+
DL-Glycerate		d	+	+	+	+	-	d	d	+	d
Glycolate		-	-	-	-	-	-	-	-	+	-
m-Hydroxybenzoate		+	-	-	-	-	-	-	-	-	-
p-Hydroxybenzoate		+	+	d	+	+	d	-	-	+	d
DL-3-Hydroxybutyrate		+	d	+	d	+	d	-	d	+	d
Isobutyrate		-	-	-	-	-	-	-	d	d	-
L-Isoleucine	+	-	-	-	d	d	-	-	+	d	+
2-Ketogluconate		+	-	+	+	-	d	-	+	-	-
5-Ketogluconate		-	-	+	+	-	-	-	-	-	-
2-Ketoglutarate		d	+	-	d	-	d	d	-	-	-
DL-Lactate	-	+	+	+	+	+	+	+	+	+	+

(continued)

TABLE BXII.α.113. (cont.)

Substrate ^{c,d}	Rhizobium leguminosarum	Rhizobium galegae	Rhizobium tropici	Agrobacterium tumefaciens	Agrobacterium rhizogenes	Agrobacterium vitis	Allorhizobium undicola	Sinorhizobium fredii	Sinorhizobium meliloti	Sinorhizobium saheli	Sinorhizobium terangae
L-Leucine	+	d	d	d	d	-	-	d	d	d	+
L-Lysine	+	d	-	-	+	-	-	-	+	+	+
D-Lyxose		+	+	+	+	+	+	-	+	d	d
Malonate		-	d	-	-	-	-	-	-	-	-
D-Mandelate		-	+	-	-	-	-	-	d	-	-
D-Melibiose		+	+	-	+	+	-	+	+	+	+
D-Melezitose		-	+	+	-	-	-	d	+	d	d
Methyl-D-glycoside		d	+	-	+	-	-	-	+	-	-
Methyl-D-xyloside		d	+	+	+	-	-	+	+	+	+
L-Ornithine		d	d	-	+	-	+	d	-	d	-
L-Phenylalanine	+	+	+	-	-	-	-	-	+	+	+
Propionate		-	+	-	+	+	(+)	d	+	+	(+)
Pyruvate	-	+	+	+	+	+	-	+	+	+	+
D-Raffinose		+	+	+	+	+	d	+	+	d	d
Salicin		-	-	-	+	+	-	-	+	-	-
Sarcosine		+	+	-	+	d	+	-	-	+	d
L-Serine	+	-	d	d	+	-	-	-	+	-	-
L-Sorbose		-	-	+	+	-	-	-	+	-	-
D-Tagatose		-	-	+	+	-	-	-	-	-	-
D-Tartrate		-	-	+	-	-	-	-	-	-	-
L-Tartrate		-	+	+	-	+	-	-	-	-	-
meso-Tartrate		-	-	+	-	-	-	-	-	-	-
L-Threonine	+	d	d	-	+	d	+	-	d	+	d
Trigonelline		-	+	-	d	+	d	d	+	d	-
L-Tyrosine	+	+	-	-	-	-	-	-	d	d	d
L-Valine	+	-	-	-	d	-	-	-	d	+	d
Xylitol		+	+	+	+	-	-	-	+	-	+
L-Xylose		-	+	d	+	d	-	-	d	d	d

^aFor symbols see standard definitions; (+), weak reaction.^bCarbon source utilization data is from de Lajudie et al. (1994) and de Lajudie et al. (1998a). Data for *R. leguminosarum* are from Amarger et al. (1997).^cSubstrates which gave positive reactions by strains of all species: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, L-histidine, inositol, D-mannose, rhamnose, ribose, sorbitol, D-turanose, and D-xylose. Fumarate, glycerol, lactose, L-malate, maltose, mannitol, sucrose, succinate, and trehalose were substrates for which only one species expressed variable negative reactions.^dSubstrates which gave negative reactions by strains of all species: adipate, D-("alpha")-alanine, DL-2-aminobutyrate, anilamine, azelate, benzoate, benzylamine, caprate, *n*-caprate, caprylate, citraconate, diammonobutane, esculin, ethylamine, glycine, glycerol, heptanoate, histamine, *o*-hydroxybenzoate, inulin, isophthalate, isovalerate, itaconate, DL-kynurenine, levulinate, maleate, mesaconate, L-methionine, DL-norvaline, oxalate, pelargonate, phenylacetate, phthalate, pinelate, sebacate, spermine, starch, suberate, terephthalate, D-tryptamine, tryptophan, urea, and *n*-valerate. 2-Aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, L-cysteine, fumarate, methyl-D-mannoside, L-norleucine, and sucrose were substrates for which only one species expressed variable positive reactions.^eData are included only where information is available for two or more strains.

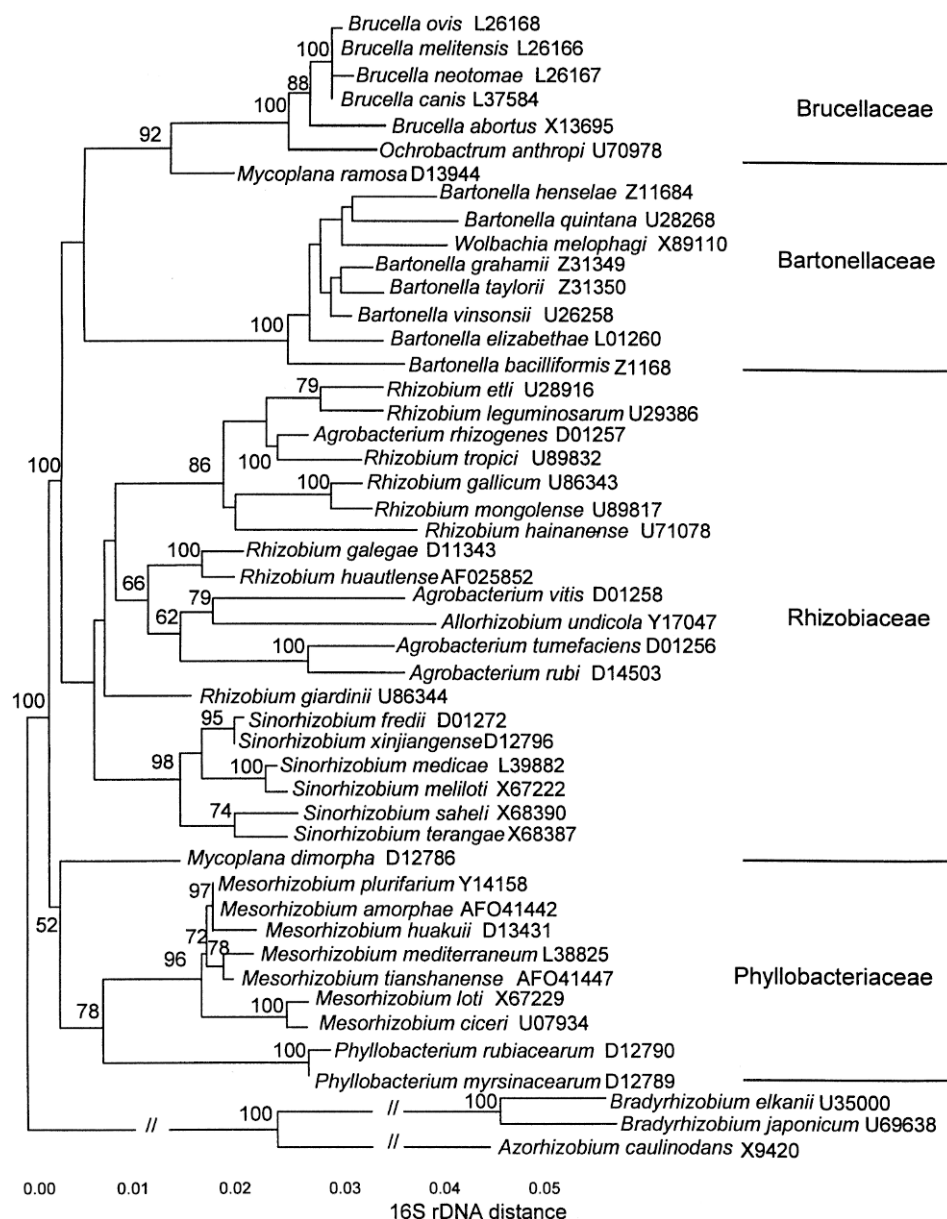


FIGURE BXII.α.130. Neighbor-joining tree expressing the relationships of genera within the *Rhizobiaceae* (*Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium* and related families) based on 16 S rDNA sequences. Bootstrap probabilities (as percentages) are determined from 1000 resamplings.

when there is clear justification based on a unique circumscription of the taxon, the practice of naming taxa when they are merely outlying members of larger clades must be questioned. In such cases, where the description of the genus is indistinguishable from adjacent genera and where the species description forms the basis of the generic description, there is no basis for forestalling an on-going process of naming monospecific genera across the clade. This approach to classification implies the creation of many genera containing small numbers of species with circumscriptions indistinguishable from *Rhizobium*.

In regard to "rhizobia," legume plants are represented by more than 14,000 species (Jordan 1984a; Lindström et al., 1998), of which fewer than 1% have been investigated to establish the identity of their associated nitrogen-fixing bacterial species. Considering the relatively close relationships of taxa within the *Rhi-*

zobiaceae, it is highly probable that new taxa will be characterized that will be intermediate between the present named species and genera (*Agrobacterium*, *Rhizobium*, and *Sinorhizobium*). Intermediate taxa can be expected to obscure the apparent deep branches between currently named genera (Martínez-Romero and Caballero-Mellado, 1996).

Murray et al. (1990) have made clear the expectation that at the generic level, taxa should be supported with phenotypic descriptions. Proposed minimal standards for *Agrobacterium* and *Rhizobium* require that generic as well as species names should be based on both phenotypic and phylogenetic data (Graham et al., 1991). Phylogenetic divisions can only be understood as generating distinct genera where these have evolved into discrete phenotypic groups. Genera differentiated solely on sequence data have the same nomenclatural status as taxa erected as *Candidatus*,

as described by Murray and Stackebrandt (1995). Furthermore, 16S rDNA sequence data cannot be accepted uncritically where there is evidence of recombination within sequences (Eardly et al., 1996; Martínez-Romero and Caballero-Mellado, 1996; Young and Haukka, 1996).

With the exception of the 16S rDNA-based discrimination of *Sinorhizobium* as a distinct clade, the generic circumscriptions of *Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium* contain no demonstrated characteristics by which these genera can be supported as distinct taxa. Multiple character analysis towards a phenetic (Goodfellow and O'Donnell, 1993; Goodfellow et al., 1997) or polyphasic (Vandamme et al., 1996b) classification aims to produce coherent taxa with relevant circumscriptions. Electrophoretic protein patterns, and numerical analysis of nutritional and biochemical data (de Lajudie et al., 1994) gave no support for segregation of species into the separate genera *Agrobacterium*, *Rhizobium*, and *Sinorhizobium*. Terefework et al. (1998) and de Lajudie et al. (1998a) provide data showing that various pathogenic and nodulating, symbiotic bacteria belonging to the genera *Agrobacterium* and *Rhizobium* are interspersed on subbranches of 16S or 23S phylogenetic trees. Analysis of fatty acid profiles showed that these three genera were closely related (Jarvis et al., 1996), but that species of *Mesorhizobium* formed a distinct group. More recently, Tighe et al. (2000) showed that the concentration of C_{16:0 3OH} fatty acid was generally somewhat lower in *Sinorhizobium* than *Rhizobium*. 16S rDNA sequence data clearly show that *Agrobacterium* spp. (Willems and Collins, 1993) are closely related to *Rhizobium* spp. such as *Rhizobium galegae* (Lindström, 1989), and the recently described *Rhizobium huautlense* (Wang et al., 1998). *Agrobacterium rhizogenes* is always found among authentic *Rhizobium* spp. Many past studies have suggested the

need for amalgamation of *Agrobacterium* and *Rhizobium* (Graham 1964, 1976; Heberlein et al., 1967; De Ley, 1968; White, 1972; Kerr, 1992; Sawada et al., 1993). It has long been clear that pathogenic (*Agrobacterium*) and nitrogen-fixing (*Rhizobium*) species are interspersed, and there seems to be a paucity of justification for the on-going separation of these genera. *Agrobacterium* is a polyphyletic genus that is an artificial amalgamation of plant-pathogenic species (Young et al., 2001).

The four named genera—*Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium*—are closely related in genomic and phenotypic terms. It has recently been proposed by the authors of this treatment that *Agrobacterium*, *Allorhizobium*, and *Rhizobium* be amalgamated into a single genus, *Rhizobium*, based on their close genomic and phenotypic similarity (Young et al., 2001). We maintain that *Allorhizobium* is an artificial genus and in phylogenetic terms is part of the *Agrobacterium*/*Rhizobium* cluster. Indeed it seems clear that its creation was only justified based on the anomalous state of *Agrobacterium* nomenclature at the time it was proposed by de Lajudie et al., (1998a). The branches between *Agrobacterium* and *Rhizobium* on the one hand and *Sinorhizobium* on the other suggest an evolutionary divergence that could be interpreted as preliminary to the formation of new genera. At present these two clades are probably incipient genera and merit no more than recognition as subgenera.²

2. Since the completion of this manuscript, a proposal to include species of *Agrobacterium*, *A. radiobacter*, *A. rhizogenes*, *A. rubi*, *A. vitis*, and *Allorhizobium undicola* in *Rhizobium* with an emended description of the genus, has been made (Young et al., 2001), as a more natural polyphasic, interpretation of the taxonomy of the family *Rhizobiaceae*. The status of the genus *Sinorhizobium* is considered to need further evaluation.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHIZOBIUM*

Morphological, biochemical, and nutritional data are given in Tables BXII.α.112 and BXII.α.113. Nutritional data have been reported for only about half of *Rhizobium* species (Table BXII.α.113). Data are included here only if more than one strain for each species has been reported. Some recently named species have been established largely using comparative 16S rDNA sequence analyses. Phenotypic descriptions of these species, as well as a comprehensive comparative investigation of the whole genus, are needed.

The phenotypic descriptions of the symbiotic nodule-forming

Rhizobium species are based on D.C. Jordan's treatment in the first edition of *Bergey's Manual of Systematic Bacteriology* in 1984 and on original descriptions of new species. The phenotypic description of *Agrobacterium* is based on studies by De Ley et al. (1966), Lippincott and Lippincott (1969), Keane et al. (1970), White (1972), Kersters et al. (1973), Panagopoulos and Psallidas (1973), Kerr and Panagopoulos (1977), Süle (1978), Panagopoulos et al. (1978), Holmes and Roberts (1981), Sawada and Ieki (1992b), de Lajudie et al. (1994), and Amarger et al. (1997).

List of species of the genus *Rhizobium*

1. ***Rhizobium leguminosarum*** (Frank 1879) Frank 1889, 338^{AL} (*Schinzia leguminosarum* Frank 1879, 397.)
le.gu.mi.no.sa'rum. M.L. fem. n. *Leguminosae* old family name of the legumes; M.L. gen. pl. n. *leguminosarum* of legumes.

The characteristics are as given for the genus and listed in Tables BXII.α.112 and BXII.α.113. The cells have either 1 or 2 polar flagella or 2–6 peritrichous flagella. Growth does not occur at 39–40°C. The pH range for growth is 4.5–9.0. Growth does not occur in YMA containing 2% NaCl. Pantothenate and, for some strains, thiamine are required as growth factors. Well characterized genetically. Three biovars of *R. leguminosarum* have been defined, biovar *trifolii* (previously classified as *Rhizobium trifolii* Dangeard 1926^{AL}), biovar *phaseoli*, and biovar *viceae*, based on nodulating specificity. Selected strains of biovar *phaseoli* have been elevated to the status of species as *R. etli*, *R. gallicum*, *R.*

giardinii, *R. mongolense*, and *R. tropici*. Residual strains representing *R. leguminosarum* need to be re-examined and the description of the species needs to be emended.

R. leguminosarum nodulates with some, but not necessarily all, *Lathyrus* spp., *Lens* spp., temperate species of *Phaseolus* (*P. vulgaris*, *P. angustifolius*, *P. multiflorus*), *Pisum* spp., *Trifolium* spp., and *Vicia* spp.

The mol% G + C of the DNA is: 59–63 (*T_m*).

Type strain: ATCC 10004, DSM 30132, NCIB 11478, USDA 2370.

GenBank accession number (16S rRNA): U29386.

2. ***Rhizobium etli*** Segovia, Young, and Martínez-Romero 1993, 376^{VP}
et'li. L. n. *etli* bean; N.L. gen. n. *etli* of bean.

The characteristics are as given for the genus and listed

in Table BXII.α.112. Fast growing: colonies are 2–4 mm in diameter after 2–4 d on peptone–yeast extract agar. No growth occurs on Luria broth medium or on peptone yeast-extract medium lacking calcium. Growth occurs on a minimal medium containing malate as a carbon source. Maximum temperature for growth is 37°C. Selected strains of *R. leguminosarum* biovar phaseoli were differentiated as this new species on the basis of differences in protein profiles, antibiotic resistance profiles, serological types, DNA–DNA reassociation data, plasmid profiles, exopolysaccharide structures, and multilocus enzyme electrophoresis.

The species contains two named biovars: *Rhizobium etli* biovar phaseoli and biovar mimosae (Wang et al., 1999a). The species nodulates and fixes nitrogen in association with *Phaseolus vulgaris* and some other legumes, such as *Mimosa affinis*. Nonsymbiotic strains are included in the species.

The mol% G + C of the DNA is: 59–63 (T_m).

Type strain: CFN 42, ATCC 51251, ICMP 13642, USDA 9032.

GenBank accession number (16S rRNA): U28916.

3. *Rhizobium galegae* Lindström 1989, 365^{VP}

ga.le'gae. M.L. fem. gen. n. *galegae* of *Galega*, a genus of leguminous plants.

The characteristics are as given for the genus and listed in Tables BXII.α.112 and BXII.α.113. Motile by 1–2 polar or subpolar flagella. Relatively slow growing. Colonies on YMA are more than 1.0 mm in diameter after 7 d at 28°C. Growth does not occur on YMA containing 2% NaCl. Maximum temperature for growth is 33–37°C. Most strains form a serum zone and give an alkaline reaction in litmus milk. Hydrolyzes urea but does not precipitate calcium glycerophosphate or reduce nitrate. Requires pantothenate as a vitamin supplement or growth factor, but not thiamine. Utilizes relatively few organic substrates as sole sources of carbon (Table BXII.α.113). A preliminary report gave information on this species (Lindström and Lehtomäki, 1988). Nodulates *Galega orientalis* and *Galega officinalis* and is reported to be specific to these species.

The mol% G + C of the DNA is: 63 (T_m).

Type strain: HAMBI 540, ATCC 43677, DSM 11542, ICMP 13643, LMG 6214.

GenBank accession number (16S rRNA): D11343, X67226.

4. *Rhizobium gallicum* Amarger, Macheret, and Laguerre 1997, 1005^{VP}

gal'li.cum. L. adj. *gallicum* pertaining to Gallia; the country of origin, France.

The characteristics are as given for the genus and listed in Table BXII.α.112. Relatively fast growing. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Growth does not occur on YMA containing 1% NaCl. Resistant to nalidixic acid. Separated from other *Rhizobium* species by cluster analysis of phenotypic data. The status of *R. gallicum* as an authentic species is supported by amplified 16S rDNA restriction analysis, comparative 16S rDNA sequence analysis, DNA–DNA reassociation, and nutritional data.

Two biovars, *R. gallicum* biovar gallicum and *R. gallicum* biovar phaseoli, are established based on nodulating specificity. *R. gallicum* biovar gallicum nodulates and fixes nitrogen in association with *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Onobrychis viciifolia*, and *Phaseolus* spp.

whereas *R. gallicum* biovar phaseoli nodulates *Phaseolus* spp. only.

The mol% G + C of the DNA is: not available.

Type strain: R602sp, MSDJ1109.

GenBank accession number (16S rRNA): AF008130, U86343.

5. *Rhizobium giardinii* Amarger, Macheret, and Laguerre 1997, 1005^{VP}

giar.di'ni.i. N.L. gen. n. *giardinii* of Giardini, a Brazilian microbiologist who isolated the organism.

The characteristics are as given for the genus and listed in Table BXII.α.112. Relatively fast growing. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Growth does not occur on YMA containing 2% NaCl. Resistant to nalidixic acid. The status of *R. giardinii* as an authentic species is supported by amplified 16S rDNA restriction analysis, DNA–DNA hybridization, and comparative 16S rDNA sequence analysis.

Two biovars, *R. giardinii* biovar giardinii and *R. giardinii* biovar phaseoli, are established based on nodulating specificity. *R. giardinii* biovar giardinii nodulates *Phaseolus* spp., *Leucaena leucocephala*, and *Macroptilium atropurpureum*, but does not fix nitrogen with *Phaseolus vulgaris*. *R. giardinii* biovar phaseoli nodulates *Phaseolus* spp. and is weakly efficient in fixing nitrogen in association with that host.

The mol% G + C of the DNA is: not available.

Type strain: H152, MSDJ0144.

GenBank accession number (16S rRNA): U86344.

6. *Rhizobium hainanense* Chen, Tan, Gao, Li, and Wang 1997b, 872^{VP}

hai.na.nen'se. M.L. neut. adj. *hainanense* pertaining to Hainan Province in China.

The characteristics are as given for the genus and listed in Table BXII.α.112. Motile by a single polar flagellum. Relatively fast growing, with a generation time of 2–4 h. Colonies are 2–4 mm in diameter after 3 d growth on yeast extract mannitol agar. Temperature optimal for growth, 25–30°C. Strains can grow at 40°C. Optimal pH for growth, 6–8; pH range, 5–10. Grows on YMA containing 2% NaCl.

The status of *R. hainanense* as an authentic species is supported by cluster analysis of phenotypic features, DNA–DNA reassociation data, and comparative 16S rDNA sequence analysis.

Nodulates *Acacia sinicus*, *Arachis hypogaea*, *Centrosema pubescens*, *Desmodium gyroides*, *D. sinuatum*, *D. triquetrum*, *D. heterophyllum*, *Macroptilium lathyroides*, *Stylosanthes guianensis*, *Tephrosia candida*, *Uraria crinita*, and *Zornia diphylla*.

The mol% G + C of the DNA is: 59–63 (T_m).

Type strain: I66, CCBAU 57015, DSM 11917, ICMP 13690.

GenBank accession number (16S rRNA): U71078.

7. *Rhizobium huautlense* Wang, van Berkum, Beyene, Sui, Dorado, Chen and Martínez-Romero 1998, 696^{VP}

hu.aut.len'se. N.L. adj. *huautlense* of Huautla, the region in Mexico where the organisms were isolated.

The characteristics are as given for the genus and listed in Table BXII.α.112. Relatively fast growing, with a generation time of 2.0–2.2 h. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Grows at 40°C. Growth does not occur on YMA containing 2% NaCl. Requires thiamine as a growth factor. Comparative sequence analysis of 16S rDNA indicates that this species is closely related to *R. galegae*. These

two species are differentiated based on multilocus enzyme electrophoresis, DNA–DNA reassociation, size of compatible Sym plasmids, and a small number of other features. Nodulates *Sesbania herbacea*, *S. rostrata*, and *Leucaena leucocephala*.

The mol% G + C of the DNA is: 57–59 (T_m).

Type strain: S02, ICMP 13551, USDA 4900.

GenBank accession number (16S rRNA): AF025852.

8. **Rhizobium lupini** (Schroeter 1886) Eckhardt, Baldwin and Fred 1931, 273^{AL} (*Phytomyxa lupini* Schroeter 1886, 135.) *lu.pi'ni*. M.L. masc. n. *Lupinus* generic name of lupine; M.L. gen. n. *lupini* of *Lupinus*.

A limited description is given in Jordan and Allen (1974). Jordan (1984a) recognized the affinities of this species with *Bradyrhizobium* species but felt that evidence was lacking to propose the transfer of *R. lupini* to the genus. Since then comparative sequence analyses of 16S rDNA from nodulating strains of *Lupinus* support transfer of the species; however, the 16S rDNA sequence of the type strain, which has unfortunately been contaminated with *B. japonicum* but still contains another distinct species (van Berkum et al., 1998), appears not to have been analyzed. The status of this species clearly needs work.

Nodulates *Lupinus* spp. and *Ornithopus* spp. Limited nodulation of *Glycine* spp. and the cowpea miscellany.

The mol% G + C of the DNA is: not available.

Type strain: ATCC 10319, DSM 30140.

9. **Rhizobium mongolense** van Berkum, Beyene, Bao, Campbell and Eardly 1998, 21^{VP} *mon.go.len'se*. L. neut. adj. *mongolense* pertaining to Inner Mongolia, the region where the bacteria were isolated.

The characteristics are as given for the genus and listed in Table BXII.α.112. Motile by a single polar or subpolar flagellum. Relatively fast growing: colonies 1–3 mm in diameter within 3–5 days on arabinose-gluconate agar. No growth occurs on YMA containing 1% NaCl or at pH values below 4.0. Resistant to bacitracin, cefoperazone, and pen-

icillin. *G. R. mongolense* shares 99.2% similarity in its 16S rDNA sequence with *R. gallicum* (van Berkum et al., 1998), and it may therefore be a junior synonym of *R. gallicum*. Isolated from *Medicago ruthenica*. Nodulates *Medicago ruthenica* and *Phaseolus vulgaris*.

The mol% G + C of the DNA is: not available.

Type strain: USDA 1844 (ICMP 13688).

GenBank accession number (16S rRNA): U89817

10. **Rhizobium tropici** Martínez-Romero, Segovia, Mercante, Franco, Graham and Pardo 1991, 424^{VP} *tro'pi.ci*. M.L. gen. n. *tropici* of the tropic (of Cancer).

The characteristics are as given for the genus and listed in Tables BXII.α.112 and BXII.α.113. Motile by peritrichous flagella. Relatively fast growing, with a doubling time of 1.6–2.0 h. Colonies are 2–4 mm in diameter within 2–4 days at 30°C on mannitol–yeast extract agar and peptone yeast-extract agar. Can grow at 40°C. Growth occurs at pH 4.5–7. Does not produce 3-ketolactose. Utilizes a range of organic substrates as sole sources of carbon (Table BXII.α.113).

Previously classified as Type II strains of *Rhizobium leguminosarum* biovar phaseoli. This species comprises Type A and Type B strains, which may represent two distinct species. Distinguished from *Rhizobium leguminosarum* by host range, *nif* gene organization, high temperature tolerance, and extreme acid tolerance, and are said to be more symbiotically stable. Distinguished from other *Rhizobium* species by DNA–DNA reassociation, multilocus enzyme electrophoresis profiles, in biochemical tests, and 16S rDNA sequence comparison.

Forms nodules on *Phaseolus vulgaris*, *Leucaena* spp., and with other legume species. Type A strain CFN299 also nodulates *Amorpha fruticosa*.

The mol% G + C of the DNA is: 60–62 (T_m).

Type strain: ATCC 49672, ICMP 13646, IFO 15427, LMG 9503, USDA 9030.

GenBank accession number (16S rRNA): U89832, X77125.

Genus II. *Agrobacterium* Conn 1942, 359^{AL*}

JOHN M. YOUNG, ALLEN KERR AND HIROYUKI SAWADA

A.gro.bac.te'ri.um. Gr. n. *agros* a field; Gr. dim. neut. n. *bakterion* a small rod; M.L. neut. n. *Agrobacterium* a small field rod.

Rods 0.6–1.0 × 1.5–3.0 μm, occurring singly or in pairs. Non-sporeforming. Gram negative. **Motile by 1–4 peritrichous flagella.** **Aerobic**, possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Some strains are capable of anaerobic respiration in the presence of nitrate. Most strains are able to grow under reduced oxygen tensions in plant tissues. Optimal temperature for growth: 25–28°C. Colonies are usually convex, circular, smooth, nonpigmented to light beige. **Growth on carbohydrate-containing media is usually accompa-**

nied by copious extracellular polysaccharide slime. Catalase positive. **Usually oxidase positive** and urease positive. Indole is not produced. Chemooorganotrophs, utilizing a wide range of carbohydrates, salts of organic acids, and amino acids as carbon sources, but not cellulose, starch, agar, or chitin. **Produce an acid reaction in mineral salts media containing mannitol and other carbohydrates.** Ammonium salts and nitrates can serve as nitrogen sources for strains of some species; others require amino acids and additional growth factors. 3-ketoglycosides are produced by the majority of strains belonging to *A. tumefaciens*. **Strains of some species in this genus invade the crown, roots, and stems of a great variety of dicotyledonous and some gymnospermous plants via wounds, causing transformation of the plant cells into autonomously proliferating tumor cells. Onco-**

*Editorial Note: Young et al. (2001) have proposed the transfer of the type species of the genus *Agrobacterium*, *A. tumefaciens*, as well as the species *A. rhizogenes*, *A. rubi*, and *A. vitis* to the genus *Rhizobium*.

genicity is correlated with the presence of a large tumor-inducing plasmid. Habitat: soil. Oncogenic strains occur mainly in soils previously contaminated with diseased plant material. Some non-oncogenic *Agrobacterium* strains have been isolated from human clinical specimens.

The mol% G + C of the DNA is: 57–63.

Type species: *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942, 359^{AL} (*Bacterium tumefaciens* Smith and Townsend 1907, 672; *Agrobacterium radiobacter* (Beijerinck and van Delden 1902) Conn 1942, 359; *Agrobacterium radiobacter* biovar radiobacter (Beijerinck and van Delden 1902) Keane, Kerr and New 1970, 594; *Agrobacterium radiobacter* biovar tumefaciens (Smith and Townsend 1907) Keane, Kerr and New 1970, 594; *Agrobacterium radiobacter* pathovar tumefaciens (Smith and Townsend 1907) Young, Dye, Bradbury, Panagopoulos and Robbs 1978, 156.)

FURTHER DESCRIPTIVE INFORMATION

Introductory note on nomenclature used in this chapter Following Bradbury (1986), strains in past literature called *Agrobacterium* biovar 1 are herein referred to as *A. tumefaciens*. *Agrobacterium* biovar 2 strains are referred to as *A. rhizogenes*. *Agrobacterium* biovar 3 strains are referred to as *A. vitis*. Where necessary, the ability of pathogenic strains to cause crown gall tumors or the hairy root condition, previously attributed to strains using the names “*Agrobacterium tumefaciens*” and *A. rhizogenes*, is indicated by reference to the tumorigenic or rhizogenic capacity respectively of strains in species of *Agrobacterium*. Nonpathogenic strains previously named *A. radiobacter* are referred to as nonpathogenic strains of *A. tumefaciens* and *A. rhizogenes* or as nonpathogenic *Agrobacterium* if the species designation has not been identified. Ti or Ri plasmids determine the pathogenic status of strains (See below). Species comprising pathogenic or nonpathogenic strains are reported as tumorigenic as a (Ti strain) or (Ti); as rhizogenic as a (Ri strain) or (Ri); or as nonpathogenic strains of the species where relevant. Table BXII.α.114 explains the relationships of names in the literature.

Acid is produced in mineral salts media from L-arabinose, cellobiose, D-fructose, D-glucose, lactose, maltose, melezitose, L-rhamnose, trehalose, D-xylose, adonitol, arabitol, ethanol, mannitol, salicin, and other carbohydrates.

Plant diseases associated with agrobacteria are commonly known as crown gall, hairy root, cane gall, and grapevine gall. Strains of some species possess a wide host range (*A. rhizogenes* and *A. tumefaciens*), whereas others (*A. rubi* and *A. vitis*) possess a limited host range, perhaps confined to single plant genera. The tumors are self-proliferating and can be transmitted by grafting.

Tumor induction is correlated with the presence of a large tumor-inducing plasmid (Ti-plasmid) in the bacterium. Hairy root induction is associated with the presence of an Ri plasmid. Both plasmids are involved in similar mechanisms of symptom production. Some strains lack oncogenic plasmids and are nonpathogenic.

The molecular size of the *Agrobacterium* genome ranges from 3.0 to 3.6×10^9 bp.

Additional information about agrobacteria can be found in the chapter on the genus *Rhizobium*.

ENRICHMENT AND ISOLATION PROCEDURES

Several selective media have been described for the isolation of *Agrobacterium* species from soil and crown gall tissues (Moore et al., 1988). The medium of Schroth et al. (1965)¹ is a general

medium for the isolation of *A. tumefaciens*. Medium 1A² of Brisbane and Kerr (1983) can be used for most *Agrobacterium tumefaciens* strains. Medium 2E of Brisbane and Kerr (1983)³ is used for *Agrobacterium rhizogenes*; erythritol was selected as the sole carbon source in this medium because *Agrobacterium tumefaciens* strains cannot utilize it (see Table BXII.α.113 in the chapter on the genus *Rhizobium*). Agrobacteria can be isolated from soil and from young crown gall tissues by spreading 0.1 ml of the appropriate dilution of soil or extracts of gall tissue with an L-shaped glass rod over one of the surface-dried media in Petri dishes, which are subsequently incubated at 27°C. Typical convex, glistening, and circular colonies with an entire edge are transferred for storage and identification.

Other selective media were described by Clark (1969), Kado and Heskett (1970), and Moore et al. (1980). A selective medium⁴ has been developed for *Agrobacterium vitis* (Roy and Sasser 1983).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Pathogenicity No single host plant will serve for the assay of virulence of tumorigenic strains of *Agrobacterium* because the host range of some strains is restricted (Panagopoulos and Psallidas, 1973; Anderson and Moore, 1979) (see above). Sunflower (*Helianthus annuus*) may be host to the widest range of tumorigenic strains. Young vigorously growing plants are recommended. Stems of 1–2 week-old sunflower plants are inoculated with sterile needles or sharpened sterile toothpicks dipped in colonies or heavy ($>10^8$ cfu/ml) aqueous bacterial suspensions prepared from 2-d-old cultures on any medium producing satisfactory growth. Pathogenicity can usually be recorded within a week after inoculation. The young stems of various varieties of the following plants can also be used: tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*) and *Kalanchoë daigremontiana* (Anderson and Moore, 1979). Tumor formation can be rather slow, taking from 2–4 weeks. As positive and negative controls, wounded plants should be inoculated with known pathogenic and nonpathogenic *Agrobacterium* strains. Inoculated plants should be

1. The medium of Schroth et al. (1965) consists of (g/l of distilled water): agar, 20.0; mannitol, 10.0; NaNO₃, 4.0; MgCl₂, 2.0; calcium propionate, 1.2; MgHPO₄·3H₂O, 0.2; MgSO₄·7H₂O, 0.1; NaHCO₃, 0.075; and magnesium carbonate, 0.075. The pH is adjusted to 7.1 with 1 N HCl. After the medium is autoclaved and cooled to 50–55°C, the following compounds are added aseptically to give final concentrations of (mg/l): berberine, 275; sodium selenite, 100; penicillin G (1625 U/mg), 60; streptomycin sulfate, 30; cycloheximide, 250; tyrothricin, 1.0; and bacitracin (65 U/mg), 100.

2. Medium 1A of Brisbane and Kerr (1983) contains (g/l of distilled water): L (–) arabitol, 3.04; NH₄NO₃, 0.16; KH₂PO₄, 0.54; K₂HPO₄, 1.04; MgSO₄·7H₂O, 0.25; sodium taurocholate, 0.29; crystal violet (0.1% w/v aqueous), 2 ml; and agar, 15.0. Autoclave; cool to 50°C, then add 1 ml of a filter-sterilized 2% solution of cycloheximide and 6.6 ml of a filter-sterilized 1% solution of Na₂SeO₃.

3. Medium 2E for *Agrobacterium rhizogenes* strains (Brisbane and Kerr, 1983) consists of (g/l of distilled water): NH₄NO₃, 0.16; erythritol, 3.05; KH₂PO₄, 0.54; K₂HPO₄, 1.04; MgSO₄·7H₂O, 0.25; sodium taurocholate, 0.29; yeast extract (1% w/v aqueous), 1 ml; malachite green (0.1% w/v aqueous), 5 ml; agar, 15. Autoclave; cool to 50°C, then add 1 ml of a filter-sterilized 2% solution of cycloheximide and 6.6 ml of a filter-sterilized 1% solution of Na₂SeO₃.

4. The medium for *Agrobacterium vitis* of Roy and Sasser (in Moore et al., 1988) contains (g/l): adonitol, 4.0; KH₂PO₄, 0.7; K₂HPO₄, 0.9; MgSO₄·7H₂O, 0.2; NaCl, 0.2; H₃BO₃, 1.0; yeast extract, 0.14; agar, 15; chloranthanil (Bravo 500; 4% aqueous), 0.5 ml. Adjust to pH 7.2, autoclave; cool to 50°C and add the following as filter-sterilized solutions: triphenyltetrazolium chloride, 80 mg; D-cycloserine, 20 mg; trimethoprim (in acidified water), 20 mg. Colonies of *Agrobacterium vitis* have a dark red center with white margins. Comparison with an authentic strain is recommended.

TABLE BXII.α.114. Relationships between different proposed nomenclature for the genus *Agrobacterium*

Species names based on natural classification		Species names based on pathogenicity	
Names used in this text (after Holmes and Roberts, 1981; Bradbury, 1986; Holmes, 1988)	After Keane et al. (1970); New and Kerr (1972); Kerr and Panagopoulos (1977); Panagopoulos et al. (1978)	Allen and Holding, 1974; Approved Lists (Skerman et al., 1980)	After Kersters and De Ley, 1984a
<i>A. tumefaciens</i> (Ti strain or Ti)	<i>A. radiobacter</i> ^a biovar tumefaciens (biotype 1)	<i>A. tumefaciens</i>	<i>A. tumefaciens</i> (biovar 1)
<i>A. tumefaciens</i> (Ri strain or Ri)	<i>A. radiobacter</i> biovar rhizogenes (biotype 1)	<i>A. rhizogenes</i>	<i>A. rhizogenes</i> (biovar 1)
<i>A. tumefaciens</i> (nonpathogenic)	<i>A. radiobacter</i> biovar radiobacter (biotype 1)	<i>A. radiobacter</i>	<i>A. radiobacter</i> (biovar 1)
<i>A. rhizogenes</i> (Ti strain or Ti)	<i>A. radiobacter</i> biovar tumefaciens (biotype 2)	<i>A. tumefaciens</i>	<i>A. tumefaciens</i> (biovar 2)
<i>A. rhizogenes</i> (Ri strain or Ri)	<i>A. radiobacter</i> biovar rhizogenes (biotype 2)	<i>A. rhizogenes</i>	<i>A. rhizogenes</i> (biovar 2)
<i>A. rhizogenes</i> (nonpathogenic)	<i>A. radiobacter</i> biovar radiobacter (biotype 2)	<i>A. radiobacter</i>	<i>A. radiobacter</i> (biovar 2)
<i>A. rubi</i> (Ti strain or Ti) ^b	<i>A. radiobacter</i> biovar tumefaciens (biotype 2)	<i>A. rubi</i>	<i>A. rubi</i>
<i>A. vitis</i> (Ti strain or Ti) ^b	<i>A. radiobacter</i> biovar tumefaciens (biotype 3)	<i>A. vitis</i>	<i>A. tumefaciens</i> (biovar 3)
<i>A. vitis</i> (nonpathogenic)	NR ^c	NR	NR

^aUse of the species epithet *radiobacter* in place of *tumefaciens* is now not considered acceptable in terms of the Code (Sawada et al., 1993; Bouzar, 1994).

^bOnly tumorigenic (Ti) capability has been reported for this species.

^cNR, not recorded.

kept in a greenhouse at 20–27°C. When no greenhouse facilities are available, disks of carrot roots (*Daucus carota*) are also useful (Klein and Tenenbaum, 1955; Lippincott and Lippincott, 1969). This is a convenient procedure, provided that at least 10 slices from different carrot roots are inoculated per strain and that proper controls are included, because false positive responses (cambial swellings) occasionally occur. Because pathogenic strains belonging to *A. vitis* and isolated from grapevines usually display a restricted host specificity, pathogenicity tests for these strains should be performed on the green tender shoots of grapevines (Panagopoulos and Psallidas, 1973; Panagopoulos et al., 1978).

The root-inducing ability of *A. rhizogenes* (Ri strains) is usually tested by the carrot disk assay (Lippincott and Lippincott, 1969; Moore et al., 1979) or on *Kalanchoë daigremontiana* (White and Nester, 1980b). The interpretation of such experiments is sometimes difficult because some tumorigenic strains are known to induce typical hairy root symptoms on *Kalanchoë* plants (De Cleene and De Ley, 1981).

DIFFERENTIATION OF THE GENUS *AGROBACTERIUM* FROM OTHER GENERA

See Table BXII.α.112 in the chapter on the genus *Rhizobium*. Apart from phenotypic characters that distinguish them as individual species, there are no common distinct characters that differentiate these species as separate from members of the genus *Rhizobium*.

TAXONOMIC COMMENTS

Inferred phylogenies based on comparative analyses of 16S rDNA sequence data show that plant-pathogenic *Agrobacterium* spp. are intermingled with fast-growing *Rhizobium* spp., together with *Al-lorhizobium undicola*. The genus itself is established only based on pathogenicity characteristics of its species. Apart from pathogenicity tests and nomenclatural problems posed by the confusing species epithets, the generic taxonomic relationships of *Agro-*

terium spp. are discussed fully and further descriptive information is given in the chapter on *Rhizobium*.

Nomenclatural problems *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 (type species) was the name given to strains of *Agrobacterium* capable of inducing tumorigenic reactions in many host genera (although some of these strains, isolated from *Vitis* spp., appeared to be specific to grape). *Agrobacterium rhizogenes* (Riker et al., 1930) Conn 1942 is comprised of strains capable of inducing a hairy-root (rhizogenic) reaction in host plants. *Agrobacterium radiobacter* (Beijerinck and van Del-den 1902) Conn 1942 consisted of nonpathogenic *Agrobacterium* strains, and *A. rubi* (Hildebrand 1940) Starr and Weiss 1943 contained strains capable of inducing tumorigenic reactions in *Rubus* spp. Although *A. rhizogenes* and *A. tumefaciens* comprised populations from soil and from a large number of dicotyledonous hosts, *A. rubi* was represented by isolates from *Rubus* which appeared to be specific to that host in nature. This classification was predominantly, and for some species solely, based on the phytopathogenic behavior of these bacteria. Though of practical use and widely supported as a special purpose classification, it bears no relationship to, and is inconsistent with, natural classifications of *Agrobacterium* as now understood.

When Smith and Townsend and Riker et al. first proposed the names *A. tumefaciens* and *A. rhizogenes*, they followed the custom of giving names which reported a distinctive character of the species—in this instance their pathogenic symptoms. However, the tumorigenic and rhizogenic characters are carried on different plasmids, with the consequence that the pathogenic characters are separately transmissible between species. The result is that both species *A. tumefaciens* and *A. rhizogenes* are represented by strains which may be either tumorigenic, rhizogenic, or—if lacking either kind of plasmid—nonpathogenic. Notwithstanding customary naming, in this extreme example apparently distinctive features are not characteristic of all members of the taxon, are found in these related taxa, and are mobile.

Although it was believed in the past that the different path-

ogenic reactions would be supported by physiological reactions that would justify species discriminations (Burkholder and Starr, 1948), this assumption has long been shown to be false (Young et al., 1992). Although the establishment of these species (*A. rhizogenes* and *A. tumefaciens*) based on distinct pathogenic characters was supported by Kersters and De Ley (1984a) and by subsequent workers, others have supported an alternative nomenclature based on natural classification. Holmes and Roberts (1981) and Holmes (1988) proposed a rational and consistent nomenclature for these taxonomic groups, which is used here. Bradbury (1986) also supported this classification but it has not found widespread acceptance for reasons that will be discussed below.

The classification of *Agrobacterium* species has been thoroughly studied using the following techniques: (a) numerical analysis of phenotypic characteristics (White, 1972; Kersters et al., 1973; Holmes and Roberts, 1981); (b) biochemical and physiological tests (Keane et al., 1970; Kersters et al., 1973; Kerr and Panagopoulos, 1977; Süle, 1978; Holmes and Roberts, 1981); (c) fatty acid methyl ester profiles (Sawada et al., 1992d; Jarvis et al., 1996); (d) DNA–DNA reassociation (De Ley, 1972, 1974); (e) measurements of the thermal stability of DNA–DNA hybrids (De Ley et al., 1973); and (f) comparison of electrophoretograms of soluble proteins (Kersters and De Ley, 1975). The results obtained by the above mentioned methods corroborated each other and indicated that the genus *Agrobacterium* consisted of at least three genetically and phenotypically different groups or clusters. These groups corresponded to biovars 1, 2, and 3 of Keane et al. (1970). Recently, this number has been increased to four, possibly five, groups now recognized as species (see below).

Species recognized based on their overall phenotypic and genomic relatedness are given below under the List of the species of the genus *Rhizobium* according to the type strains allocated to each species population.

Bradbury (1986), Holmes and Roberts (1981), Holmes (1988), and Young et al. (1992) have supported use of the names *A. tumefaciens* and *A. rhizogenes*, in accord with natural classification, recognizing pathogenic strains according to their tumorigenic, rhizogenic, and nonpathogenic states. However, the epithet *tumefaciens* has become so entrenched as the name for pathogenic populations of *Agrobacterium* spp. with tumorigenic capabilities in a special purpose classification, that it is difficult for it also to be used unambiguously for its proper purpose in natural classification. Attempts to resolve the difficulty by recognizing *A. radiobacter* in place of *A. tumefaciens* (Young et al., 1978; Kersters

and De Ley, 1984a; Sawada et al., 1993) cannot be adopted easily because *A. tumefaciens*, the type species, has been conserved over *A. radiobacter* (Judicial Commission, 1970). Bouzar (1994) sought clarification of the proposal of Sawada et al. (1993) which, with the response of Sawada et al. (Bouzar 1994), the Judicial Commission of the ICSB deemed to have resolved the matter (L.G. Wayne, personal communication). The practical and usual solution to this nomenclatural confusion has been to use an artificial classification and informal nomenclature in which the species names *A. tumefaciens* or *A. rhizogenes* are applied to the pathogenic plasmid-borne states, and the terms biotype or biovar are applied to the natural species groups.

If the genus *Agrobacterium* continues to be differentiated from *Rhizobium* in the future, then the use of a natural classification that recognizes *Agrobacterium* species will probably require a radical change of nomenclature by application of the Code (Lapage et al., 1992). For instance, the name *A. tumefaciens* could be rejected (Rule 23a) as a *nomen ambiguum*; a name that has been used with different meanings and has thus become a source of error (Rule 56a). Rejection would also require the designation of a new type species. The obvious candidate is *A. radiobacter*. A more radical option could involve the application of new names (and a new type species), extending the proposal of Kersters and De Ley (1984a). For both these proposals, it would be necessary to make a Request for an Opinion to the Judicial Commission of the ICSB. The four named genera—*Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium*—are closely related in genomic and phenotypic terms. It has recently been proposed that *Agrobacterium*, *Allorhizobium*, and *Rhizobium* be amalgamated into a single genus, *Rhizobium*, based on their close genomic and phenotypic similarity (Young et al., 2001) (see *Rhizobium* chapter in this volume).

Elsewhere, the application of pathovars in terms of the Standards for Naming Pathovars (Dye et al., 1980) has been proposed (Young et al., 1978; Kersters and De Ley, 1984a). However, the fact that most pathogenicity genes are carried on a plasmid means that the pathogenic character of any strain is unstable. This lack of stability would make uncertain the application of pathovar names to particular strains, most notably to pathotype strains. For pathogenic strains in *Agrobacterium* therefore, this formal special purpose nomenclature (Dye et al., 1980) seems inappropriate. Species comprising pathogenic or non-pathogenic strains could be reported as tumorigenic, as a Ti strain or Ti, as rhizogenic, as a Ri strain or Ri, or as nonpathogenic strains of the species, where relevant.

List of species of the genus *Agrobacterium*

1. ***Agrobacterium tumefaciens*** (Smith and Townsend 1907) Conn 1942, 359^{AL} (*Bacterium tumefaciens* Smith and Townsend 1907, 672; *Agrobacterium radiobacter* (Beijerinck and van Delden 1902) Conn 1942, 359; *Agrobacterium radiobacter* biovar radiobacter (Beijerinck and van Delden 1902) Keane, Kerr and New 1970, 594; *Agrobacterium radiobacter* biovar tumefaciens (Smith and Townsend 1907) Keane, Kerr and New 1970, 594; *Agrobacterium radiobacter* pathovar tumefaciens (Smith and Townsend 1907) Young, Dye, Bradbury, Panagopoulos and Robbs 1978, 156.)
tu.me.fa'ci.ens. L. n. tumor a swelling tumor; L. v. *facere* to make, to produce; M.L. part. adj. *tumefaciens* tumor producing.

The characteristics are as described for the genus and

as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Temperature optimal for growth 25–28°C and grows at 37°C. Grows in media containing 2% NaCl. Produces 3-ketolactose. Has no growth factor requirements. Utilizes a relatively wide range of organic substrates as sole sources of carbon (see Table BXII.α.113 in the chapter on the genus *Rhizobium*). The status of *A. tumefaciens* as an authentic species with the *Rhizobium* clade is supported by comparative 16S rDNA sequence analysis.

This species corresponds to biotype 1 of Keane et al. (1970), group I of White (1972), cluster 1 of Kersters et al. (1973), biovar 1 of Willems and Collins (1993) and of Sawada et al. (1993). *A. tumefaciens* comprises 3-ketolactose-positive, tumorigenic strains, as well as hairy root-forming

strains and nonpathogenic strains. It includes the type strain of *A. tumefaciens*, a tumorigenic strain, as well as the type strain of *A. radiobacter*, a nonpathogenic strain.

Ti or Ri plasmids determine the pathogenic status of strains. The species comprises pathogenic or nonpathogenic strains, which can be reported as tumorigenic, as a Ti strain or Ti, as rhizogenic as a Ri strain or Ri, or as nonpathogenic strains of the species where relevant. Pathogenic strains have a wide, and perhaps complex, host range.

An alternative special purpose nomenclature involves naming strains according to their pathogenic character, as *A. tumefaciens*, *A. rhizogenes*, or *A. radiobacter* for tumorigenic, rhizogenic and nonpathogenic strains, respectively, and classifying them as *Agrobacterium* biovar 1.

The epithet *tumefaciens* takes precedence in *Agrobacterium* because it is the designated type species of this genus.

The mol% G + C of the DNA is: 57–63 (T_m).

Type strain: ATCC 23308, DSM 30205, ICMP 5856, LMG 187, NCPPB 2437.

GenBank accession number (16S rRNA): D01256, D14500, M11223.

2. ***Agrobacterium rhizogenes*** (Riker, Banfield, Wright, Keitt and Sagen 1930) Conn 1942, 359^{AL} emend. Sawada, Ieki, Oyaizu and Matsumoto 1993, 701 (*Bacterium rhizogenes* Riker, Banfield, Wright, Keitt and Sagen 1930, 536; *Agrobacterium radiobacter* biovar *rhizogenes* (Riker, Banfield, Wright, Keitt and Sagen 1930) Keane, Kerr and New 1970, 594; *Agrobacterium radiobacter* pathovar *rhizogenes* (Riker, Banfield, Wright, Keitt and Sagen 1930) Young, Dye, Bradbury, Panagopoulos and Robbs 1978, 156.) *rhī.zō'ge.nes*. Gr. n. *rhiza* a root; Gr. v. *gennao* to make, to produce; M.L. adj. *rhizogenes* root-producing.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Temperature optimal for growth 25–28°C. Does not grow at 35°C. Does not grow in media containing 2% NaCl. Does not produce 3-ketolactose. Requires biotin as a growth factor. Utilizes a relatively wide range of organic substrates as sole sources of carbon (Table BXII.α.113). The status of *A. rhizogenes* as an authentic species in the genus *Rhizobium* is supported by numerical analysis of nutritional and biochemical data and by comparative 16S rDNA sequence analysis.

This species corresponds to biotype 2 of Keane et al. (1970), group III of White (1972), and cluster 2 of Kersters et al. (1973). It comprises 3-ketolactose-negative, tumorigenic, rhizogenic, and nonpathogenic strains.

Ti or Ri plasmids determine the pathogenic status of strains. The species comprises pathogenic or nonpathogenic strains, which can be reported as tumorigenic, as a Ti strain or Ti, as rhizogenic as a Ri strain or Ri, or as nonpathogenic strains of the species where relevant. Pathogenic strains have a wide, and perhaps complex, host range.

An alternative special purpose nomenclature involves naming strains according to their pathogenic character, as *A. tumefaciens*, *A. rhizogenes*, or *A. radiobacter* for tumorigenic, rhizogenic, and nonpathogenic strains, respectively, and classifying them as *Agrobacterium* biovar 2.

The mol% G + C of the DNA is: 59–63 (T_m).

Type strain: ATCC 11325, DSM 30148, ICMP 5794, IFO 13257, LMG 150, NCPPB 2991.

GenBank accession number (16S rRNA): D01257, D14501.

3. ***Agrobacterium rubi*** (Hildebrand 1940) Starr and Weiss 1943, 316^{AL} (*Phytomonas rubi* Hildebrand 1940, 694.) *ru'bi*. L. n. *Rubus* generic name of blackberry; L. gen. n. *rubi* of *Rubus*

The characteristics are as described for the genus and as listed in Table BXII.α.112 in the chapter on the genus *Rhizobium*. Temperature optimal for growth 25–28°C. Does not grow at 35°C (Sawada and Ieki, 1992b); grows at 37°C (Kerr, 1992). Does not grow in media containing 2% NaCl. Does not produce 3-ketolactose. Growth rate on ordinary media is characteristically slower than for the other species. Requires L-glutamic acid, biotin, pantothenate, and nicotinic acid (present in yeast extract) as growth factors. The status of *A. rubi* as an authentic species is supported by numerical analysis of nutritional and biochemical data and by comparative 16S rDNA sequence analysis.

So far, pathogenic strains of this species have been reported only from *Rubus* spp.

Tumorigenic strains bearing Ti plasmids determine the pathogenic status of strains. Isolated from aboveground cane galls on *Rubus* spp. (black raspberry, boysenberry). The natural host range is apparently limited to *Rubus* spp., but artificial inoculations indicate a wider host range (Anderson and Moore, 1979).

The mol% G + C of the DNA is: 57.6–58.8 (T_m).

Type strain: ATCC 13335, CFBP 1317, ICMP 6428, IFO 13261, LMG 156, NCPPB 1854.

GenBank accession number (16S rRNA): D14503, X67228.

4. ***Agrobacterium vitis*** Ophel and Kerr 1990, 240^{VP} *vi'tis*. L. fem. n. *vitis* wine plant; L. gen. fem. n. *vitis* of the wine plant.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Temperature optimal for growth 25–28°C. Does not grow at 35°C. Grows in medium containing 2% NaCl. Does not produce 3-ketolactose. Strains require biotin for growth. Produces acid from adonitol and mannitol, but not from alpha-methyl-D-glucoside, erythritol, dulcitol, melezitose, or arabitol. Utilizes relatively few organic substrates as sole sources of carbon (Table BXII.α.113).

The status of *A. vitis* as an authentic species is supported by numerical analysis of nutritional and biochemical data and by comparative 16S rDNA sequence analysis. The species comprises strains previously referred to as *Agrobacterium* biovar 3 *sensu* Kerr and Panagopoulos (1977) as described by Süle (1978) and Panagopoulos et al. (1978). *A. vitis* strains have been isolated from grapevines in many countries including Greece and Hungary (Panagopoulos et al., 1978), People's Republic of China (Ma et al., 1985), Australia (Ophel and Kerr, 1990), Japan (Sawada et al., 1990), France, Germany, and the United States (Otten et al., 1996), Korea, and, to date, the geographic distribution of the pathogen has reflected that of the host. Many strains of this species display a limited host range. So far, pathogenic strains of this species have been reported largely from *Vitis*

spp. although Sawada and Ieki (1992a) report an *A. vitis* strain (as biovar 3) from *Actinidia*.

Ti plasmids determine the pathogenic status of strains. The species comprises pathogenic or nonpathogenic strains, which can be reported as tumorigenic, as Ti strain or Ti, or nonpathogenic strains of the species where relevant. Strains are generally isolated from *Vitis* spp. grape,

but they have occasionally been isolated from other dicotyledonous plant species.

The mol% G + C of the DNA is: 59 (T_m).

Type strain: ATCC 49767, ICMP 10752, LMG 8750, NCPPB 3554.

GenBank accession number (16S rRNA): D01258, D14502, U45329, X67225.

Other Organisms

A small group of yellow-pigmented, 3-ketolactose-positive bacteria, including "*Chromobacterium folium*" ("*Chromobacterium lividum*") strains NCTC 10590 and 10591, that were considered to constitute another distinct group of agrobacteria (Holmes and Roberts, 1981) have since been classified as *Sphingomonas yanokuyae* (Takeuchi et al., 1995).

Marine, star-shaped, aggregate-forming bacteria named *A. ferrugineum* Ahrens and Rheinheimer 1967, *A. gelatinovorans* Ahrens 1968 (as *A. gelatinovorum*), and *A. stellulatum* Stapp and Knösel 1954 were not included in the Approved Lists. However, Rüger and Höfle (1992) reported phenotypic and genotypic data which did "not definitely support exclusion of marine strains from *Agrobacterium*." As an *ex tempore* measure they proposed these species, together with *A. atlanticum* Rüger and Höfle 1992 and *A. meteori* Rüger and Höfle 1992, as members of the genus *Agrobacterium*.

A comparative analysis of 16S rDNA sequence data has shown the marine *Agrobacterium* spp. to cluster in two subdivisions in the *Alpha*proteobacteria. *A. stellulatum* and "*A. kielense*" were in the "alpha-2" subdivision, but were more distant from *Rhizobium* than

genera of the *Bartonellaceae*, *Brucellaceae*, and *Phyllobacteriaceae*. *A. meteori*, *A. atlanticum*, *A. ferrugineum*, and *A. gelatinovorans* were in the "alpha-3" subdivision (Uchino et al., 1997). Following a reinvestigation of phenotypic characters and of inferred phylogenies based on comparative analyses of 16S rDNA sequences, Uchino et al. (1998) allocated the marine agrobacteria as follows: *Ruegeria atlantica* (Rüger and Höfle 1992) Uchino et al. 1999^{VP} (syn: *Agrobacterium meteori* Rüger and Höfle 1992), *Ruegeria gelatinovorans* (Rüger and Höfle 1992) Uchino et al. 1999 (as *R. gelatinovora*^{VP}), *Stappia stellulata* (Rüger and Höfle 1992) Uchino et al. 1999^{VP}. *Agrobacterium ferrugineum* is a member of an unidentified genus in the alpha-3 sub-group of the *Proteobacteria*.

An unnamed *Agrobacterium* species has been reported from *Ficus benjamina* (Bouzar et al., 1995). Identity as a new species is based on the presence of a unique 16S rRNA domain, on differences in nutritional profiles, and on an unusual opine metabolism. Tumorigenic and nonpathogenic strains have been isolated.

Genus III. *Allorhizobium* de Lajudie, Laurent-Fulele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus and Gillis 1998a, 1288^{VP*}

L. DAVID KUYKENDALL AND FRANK B. DAZZO

Al.lo.rhi.zo'bi.um. Gr. adj. *allos* other; M.L. neut. n. *Rhizobium* a bacterial generic name; M.L. neut. n. *Allorhizobium* the other *Rhizobium*, to refer to the fact that it is phylogenetically separate from other *Rhizobium* species.

Rods 0.5–0.7 × 2.0–4.0 µm. Nonsporeforming. Gram negative. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Relatively fast growing;** colonies 0.5–3.0 mm develop in 1–2 days on yeast–mannitol–mineral salts agar, and pronounced turbidity develops after 1–2 days in agitated broth media. Chemoorganotrophic. **A wide range of carbohydrates, organic acids, and amino acids are used as sole carbon sources for growth. Ketolactose is not produced from lactose. Growth on carbohydrate media is usually accompanied by extracellular polysaccharide production.** At the molecular level, the genus can be distinguished from related species in other genera by SDS-PAGE whole cell protein analysis, ITS PCR-RFLP, and 16S rRNA gene sequencing. **Initiates the production of root nodules, in which the bacteria occur as nitrogen-fixing intracellular symbionts.** Exhibits specificity to certain temperate-zone and tropical-zone leguminous plants (family Leguminosae).

The mol% G + C of the DNA is: 60.1.

Type species: *Allorhizobium undicola* De Lajudie, Laurent-Fu-

lele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus and Gillis 1998a, 1288.

FURTHER DESCRIPTIVE INFORMATION

Like most legume-nodulating bacteria, these organisms are Gram-negative rods but, compared with most of the fast-growing *Rhizobium*, bacteria isolated from nodules of *Neptunia natans* growing in India are even faster growing, developing approximately 2 mm colonies in 2 d on yeast mannitol agar (Vincent, 1970). Colonies are beige, round, creamy, and smooth; like most *Rhizobium* species, this organism, motile in liquid medium, also produces copious slime, which is composed of extracellular polysaccharides (de Lajudie et al., 1998a). Utilizes a wide range of carbohydrates, organic acids, and amino acids for carbon and energy sources (see Table BXII.α.113 in the chapter on the genus *Rhizobium*).

In the aquatic environment, symbiotic infection of *Neptunia* occurs by wound or "crack" entry, rather than via root hairs, followed by formation of infection threads that penetrate host nodule cells and release the endosymbionts (Subbaio-Rao et al., 1995). Thus, nodules are formed at the base of lateral and adventitious roots, wherein the bacteria occur as intracellular symbionts that fix N₂.

*Editorial Note: Young et al. (2001) have proposed the transfer of the type and only species of *Allorhizobium*, *A. undicola*, to the genus *Rhizobium*.

This species exhibits host specificity, since no isolates have been found which can nodulate *Sesbania rostrata*, *Sesbania pubescens*, *Sesbania grandiflora*, *Vigna unguiculata*, or *Macroptilium atropurpureum* (de Lajudie et al., 1998a). This species forms nodules on the temperate-zone *Medicago sativa* (alfalfa) and a number of tropical-zone (*Neptunia natans*, *Acacia senegal*, *Acacia seyal*, *Acacia tortilis* subsp. *radiana*, *Lotus arabicus*, *Faidherbia albida*) leguminous plants of the family Leguminosae (Subba-Rao et al., 1995; de Lajudie et al., 1998a).

ENRICHMENT AND ISOLATION PROCEDURES

This organism can be isolated from fresh nodules. A sample of water or soil can be used to inoculate seeds of the host plant *Neptunia natans* that have been surface-sterilized and scarified with concentrated sulfuric acid for about 5 min, followed by several rinses with sterile water. Plants are grown aseptically, so that uninoculated controls are devoid of nodules. Fahraeus plant growth medium is recommended (Fahraeus, 1957). Nodules formed are surface sterilized in 50% Clorox for a few minutes, followed by several sterile water rinses. The contents of squashed nodules can be streaked on YEM for isolation.

MAINTENANCE PROCEDURES

Lymphilized cultures stored at 4°C are recommended.

DIFFERENTIATION OF THE GENUS *ALLORHIZOBIUM* FROM OTHER GENERA

See Table BXII.α.112 in the chapter on the genus *Rhizobium*. Apart from phenotypic characters that distinguish *Allorhizobium*

undicola as an individual species, there are no common distinct characters that differentiate *Allorhizobium* from members of the genus *Rhizobium*, *Agrobacterium*, or *Sinorhizobium*.

TAXONOMIC COMMENTS

Inferred phylogenies based on comparative analyses of 16S rDNA sequence data show that *Allorhizobium undicola* is intermingled with fast-growing *Rhizobium* spp. and plant pathogenic *Agrobacterium* spp. (Young et al., 2001). Further, the circumscription of the genus does not delineate a taxon distinct from *Rhizobium* (Young et al., 2001). *Allorhizobium* is a monospecific genus established because—as indicated by its name—the data for *Allorhizobium undicola* represented an outlying branch in the particular comparative analysis of 16S rDNA sequence data (de Lajudie et al., 1998a). The closest neighboring species in this analysis is *Rhizobium vitis* (formerly *Agrobacterium vitis*). Comparison of this analysis with others that have been published (Sawada et al., 1993; Willems and Collins, 1993; de Lajudie et al., 1994, 1998b, a; Nour et al., 1995; Rome et al., 1996; Amarger et al., 1997; Chen et al., 1997b; Jarvis et al., 1997; Tan et al., 1997; van Berkum et al., 1998; Wang et al., 1998) and with Fig. BXII.α.130 of the chapter on the genus *Rhizobium* shows how much results can vary depending on the selection of sequences and upon the form of analysis. The species *R. undicola* is well defined based on DNA–DNA reassociation, PAGE of total proteins, and carbon source utilization tests. Further taxonomic comments are given in the chapter on the genus *Rhizobium*.

List of species of the genus *Allorhizobium*

1. ***Allorhizobium undicola*** De Lajudie, Laurent-Fulele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus and Gillis 1998a, 1288.
un.di' co.la. L. n. *unda* water; L. n. *cola* dweller; M.L. n. *undicola* water dweller, referring to the isolation of these strains from nodules of the aquatic plant *Neptunia natans*.

The characteristics are as described for the genus and listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Exhibits specificity to some temperate-zone (*Medicago sativa*) and some tropical-zone (*Neptunia natans*, *Acacia senegal*, *Acacia seyal*, *Acacia tortilis* subsp.

raddiana, *Lotus arabicus*, *Faidherbia albida*) leguminous plants (family Leguminosae) and incites the production of root nodules wherein the bacteria occur as intracellular symbionts. No strain has been found to nodulate *Sesbania rostrata*, *Sesbania pubescens*, *Sesbania grandiflora*, *Vigna unguiculata*, or *Macroptilium atropurpureum*. Wound or crack-entry infection of *Neptunia* occurs, rather than infection via root hairs in the aquatic environment. The closest relative is *Agrobacterium vitis* (96.2% 16S ribosomal RNA similarity).

The mol% G + C of the DNA is: 60.1 (T_m).

Type strain: ORS 992, LMG 11875.

GenBank accession number (16S rRNA): Y17047.

Genus IV. *Carbophilus* Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 393)

ORTWIN O. MEYER

car.bo.phi' lus. L. masc. *carbo* carbon; M.L. part. *philus* loving, loving carbohydrates and other carbonaceous substrates.

Rod-shaped cells with slightly tapered ends, 0.7–0.9 × 0.9–1.7 μm. Motility variable; when present, it is by means of up to five peritrichous flagella. Flagellation is maximal in the late exponential growth phase, when more than 80% of the cells are flagellated. Formation of aggregates is rare. **Gram negative**. Colonies are white to cream colored. Organic growth factors are not required. **Aerobic**, having a strictly respiratory type of metabolism, with oxygen as the terminal electron acceptor. Neither denitrification nor reduction of nitrate to nitrite has yet been observed. **Facultatively chemolithoautotrophic**. CO is utilized as a

substrate under aerobic chemolithoautotrophic conditions. Chemorganoheterotrophic substrates encompass a wide variety of the salts of organic acids (except citrate), amino acids, sugars, and sugar alcohols (see Table 1 of Meyer et al., 1993). Suitable nitrogen sources are ammonia, hydroxylamine, nitrate, and urea (Frunzke and Meyer, 1990). Dinitrogen is not fixed. Not phototrophic. Not methanogenic. Isolated from soil at the city of Moscow (Russia).

The mol% G + C of the DNA is: 62.8 (T_m).

Type species: ***Carbophilus carboxidus*** (ex Nozhevnikova and

Zavarzin 1974) Meyer, Stackebrandt and Auling 1994, 182 (Effective publication: Meyer, Stackebrandt and Auling 1993, 393.)

FURTHER DESCRIPTIVE INFORMATION

CO-oxidizing *Carbophilus carboxidus* grows with CO as energy source and CO₂ as carbon source under aerobic chemolithoautotrophic conditions, with a generation time of about 40 h (Cypionka et al., 1980). *C. carboxidus* is an exception among the aerobic CO-oxidizing bacteria in that strains capable of growing at the expense of H₂ plus CO₂ are not known. The electron transport system of *C. carboxidus* contains *b*-, *c*-, and *a*-type cytochromes at levels similar to those of other aerobic, respiratory bacteria (Cypionka and Meyer, 1983b). The *o*-type cytochrome *b*₅₃₆ serves as alternative CO-insensitive terminal oxidase.

C. carboxidus contains the megaplasmids pHCG1-a (558 kb), pHCG1-b (428 kb), and pHCG1-c (129 kb) (Kraut and Meyer, 1988). The structural genes for CO dehydrogenase (*coxLMS*) and ribulosebiphosphate carboxylase (*cbbL*) are plasmid encoded (Kraut et al., 1989).

For appropriate media, cultivation and growth conditions, type of CO dehydrogenase, and enrichment and isolation procedures, refer to the genus *Oligotropha*.

DIFFERENTIATION OF THE GENUS *CARBOPHILUS* FROM OTHER GENERA

The ability to utilize CO under aerobic chemolithoautotrophic conditions occurs only in the genera *Carbophilus*, *Oligotropha*, *Bradyrhizobium*, and *Zavarzinia*. A property that separates *Carbophilus carboxidus* from all other aerobic carboxidotrophic bacteria is its inability to grow chemolithoautotrophically on H₂ and CO₂.

The genus *Carbophilus* can be differentiated from *Oligotropha* and *Zavarzinia* by its peritrichous flagellation, the wide variety of organic substrates utilized, particularly sugars, and a different profile of fatty acids and polyamines (see Table 1 of Meyer et al., 1993). *C. carboxidus* does not fix N₂. *Carbophilus* is differentiated from *Nitrobacter* by the inability to utilize ammonium as an energy source for chemolithoautotrophic growth. It is differentiated from *Agrobacterium* by not being infectious to plants.

TAXONOMIC COMMENTS

The genus *Carbophilus* with the single species *C. carboxidus* has been allocated to the class *Alphaproteobacteria* in the phylum *Proteobacteria* (Fig. BXII.α.185 in the genus *Oligotropha*) on the basis of 16S ribosomal RNA cataloging, the presence of signature oligonucleotides in 16S rRNA catalogues (see Table 5 of Auling et al., 1988), the presence of ubiquinone Q-10 as the major quinone (see Table 3 of Auling et al., 1988), the presence of *sym*-homospermidine and putrescine as the major polyamines (see Table 4 of Auling et al., 1988), and the presence of *cis*-11,12-octadecenoic acid as the main fatty acid and 11-methyl-*cis*-11,12-octadecenoic acid as diagnostic fatty acid (see Table 3 of Auling et al., 1988). The position of *Carbophilus* is in an individual line of descent within the *Alphaproteobacteria* together with the nitrilotriacetic acid utilizing members of the genus *Chelatobacter* (Meyer et al., 1993). The 16S rRNA similarity coefficient of 0.78 separating these two taxa is lower than those found to delineate phenotypically well-described and phylogenetically coherent genera, such as *Bradyrhizobium*, *Rhizobium*, or *Nitrobacter* (Meyer et al., 1993).

List of species of the genus *Carbophilus*

1. ***Carbophilus carboxidus*** (ex Nozhevnikova and Zavarzin 1974) Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 390.)

car. box' idus. L. n. connected with carbon oxides.

The description is as given for the genus. The strain carboxydobacterium Z-1171 was isolated from soil near a

stream at Neskuchny Garden, city of Moscow, Russia; it was originally described as "*Achromobacter carboxydus*" and later as "*Alcaligenes carboxydus*" (Nozhevnikova and Zavarzin, 1974; Zavarzin and Nozhevnikova, 1976, 1977; Zavarzin, 1978; Cypionka et al., 1980; Meyer and Schlegel, 1983).

The mol% G + C of the DNA is: 62.8 (*T_m*).

Type strain: ATCC 51424, CIP 105722, DSM 1086, Z-1171.

Genus V. *Chelatobacter* Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624^{VP} (Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 109)

THOMAS W. EGLI AND GEORG AULING

Che.la'to.bac.ter. Gr. n. *chele* claw; M.L. v. *chelato* to form claw-like complexes with divalent cations, i.e., to chelate; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* rod or staff; M.L. masc. n. *Chelatobacter* chelating rod.

Rods 0.7–0.9 × 1–2 μm, often pleomorphic and budding. L-, Y-, and X-shaped forms occur. Gram negative. **Motile, usually by two or three subpolar flagella. Obligately aerobic.** Optimal growth temperature, 28–30°C; no growth at 41°C and 4°C. Slow growers with all substrates tested so far ($\mu_{\max} < 0.25 \text{ h}^{-1}$). Poly-β-hydroxybutyrate is accumulated. All strains are sensitive to β-lactam antibiotics but resistant to nalidixic acid. **The metal-chelating aminopolycarboxylic acid nitrilotriacetic acid (NTA) can be utilized as a sole source of carbon/energy and nitrogen.** A great variety of sugars, acids, alcohols, and methylated amines are utilized. Vitamins are not required. Ubiquinone Q-10 is present. The main polyamine present is *sym*-homospermidine; pu-

trescine and spermidine occur as major polyamines and spermine as a minor polyamine.

The mol% G + C of the DNA is: 62–63.

Type species: ***Chelatobacter heintzii*** Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624 (Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 110.)

FURTHER DESCRIPTIVE INFORMATION

Cell morphology All the strains isolated so far are of similar morphology, although they differ slightly with respect to their physiological properties (Egli et al., 1988). Generally the cells are motile, usually by two to three subpolar flagella (Fig. BXII.α.131a); however, for one strain (TE 10) peritrichously flag-

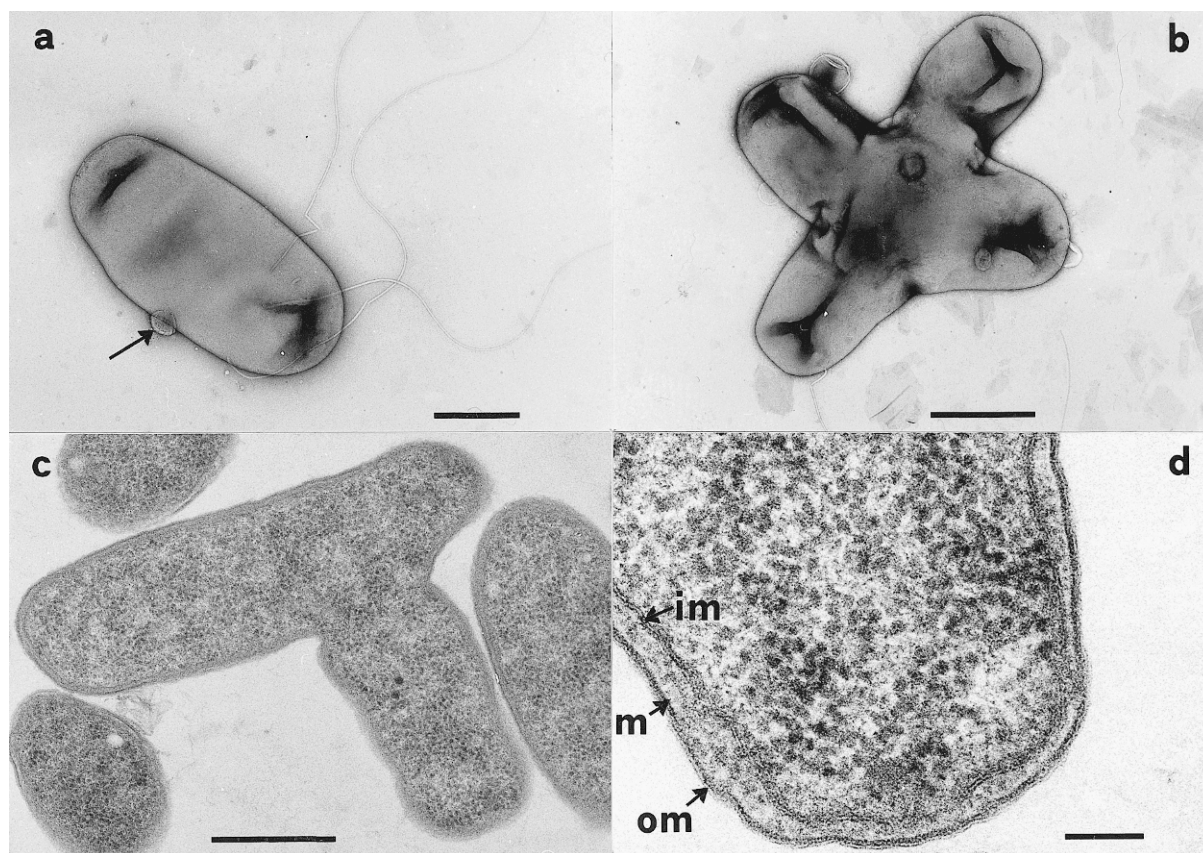


FIGURE BXII.α.131. Electron micrographs of *Chelatobacter heintzii* strains. (a), negatively stained cell of isolate TE 8 with the subpolar location of flagella and the initiation of cell budding (arrow). Bar = 0.5 μ m. (b), negatively stained cell of isolate TE 8 demonstrating pleomorphism. Bar = 0.5 μ m. (c), thin-sectioned cells of isolate TE 7 exhibiting pleomorphism. Bar = 0.5 μ m. (d), higher magnification of thin-sectioned cell of isolate TE 6 demonstrating the Gram-negative cell wall with inner membrane (im), murein layer (m), and outer membrane (om), Bar = 0.1 μ m.

ellated cells were also observed (Wehrli and Egli, 1988). The proportion of cells that are motile depends on the growth phase and substrate. In addition to cells dividing normally by binary fission, L-, Y-, and X-shaped cells are frequently detected in thin-sectioned, freeze-fractured, and negatively stained preparations (Fig. BXII.α.131b, c). In addition, bud-like structures are often observed where growth of daughter cells is obviously initiated (Fig. BXII.α.131a, b). The significance of this pleomorphism and whether or not it is dependent on nutritional or particular environmental conditions is still unknown; however, it does not result from growth with the metal-chelating compound NTA because pleomorphic cells are also observed during growth in complex media.

All isolates exhibit a peptidoglycan layer of 4–6 nm thickness (Wehrli and Egli, 1988) and an outer membrane (Fig. BXII.α.131d). The outer membrane is always closely associated with the underlying murein layer, and this is probably the reason why in freeze-fractured cells breakage occurs exclusively at the inner membrane and not at the outer membrane as well, as has been observed with most other Gram-negative bacteria (Wehrli and Egli, 1988).

Colony morphology Strains that grew well on Plate Count Agar (PCA, from Difco, Detroit, MI) exhibited round and smooth, beige-brown colonies that had a diameter of 2–3 mm after 4–5 days of growth. However, most of the strains grew faster and reliably on 0.2 \times PCA broth solidified with 1.5% agar (see

below), and on this medium the colonies of all isolates were similar: round, light-beige, slightly translucent and small in size (1.0–1.5 mm in diameter after 4–5 days). The colonies were round and volcano- or sometimes fried-egg-like, frequently with one or two concentric rings (Fig. BXII.α.132a, b, c, e). Colonies grown on NTA-containing media were white, tough, and pinpoint in size at the early stages of development (Fig. BXII.α.132b, viewed with a stereo-microscope with incident light); later a brown center developed and finally (4–5 days after streaking out) the whole colony turned black and polygonal as shown in Fig. BXII.α.132d (viewed with transmitted light).

Cultural characteristics The chemically defined medium (SM) used for isolation of *Chelatobacter heintzii* strains in both batch and chemostat culture (Egli et al., 1988; Egli and Weilenmann, 1989) contained (g/l distilled water): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.41 g; KH_2PO_4 , 0.26 g; 1 ml of the trace element stock solution described by Pfennig et al. (1981) with NTA as the chelating agent (5.2 g/l); and 1 ml of a vitamin stock solution. The vitamin solution contains (per liter): pyridoxine-HCl, 100 mg; thiamine-HCl, riboflavin, nicotinic acid, D-calcium pantothenate, *p*-amino benzoic acid, lipoic acid, nicotinamide, and vitamin B₁₂, 50 mg each; biotin, 20 mg; and folic acid, 20 mg. This medium was supplemented with either NTA or other carbon and/or nitrogen sources (up to a maximum of 5 g/l carbon). For growth with carbon sources that contained no nitrogen, the SM was supplemented with NH_4Cl , 0.54 g/l.

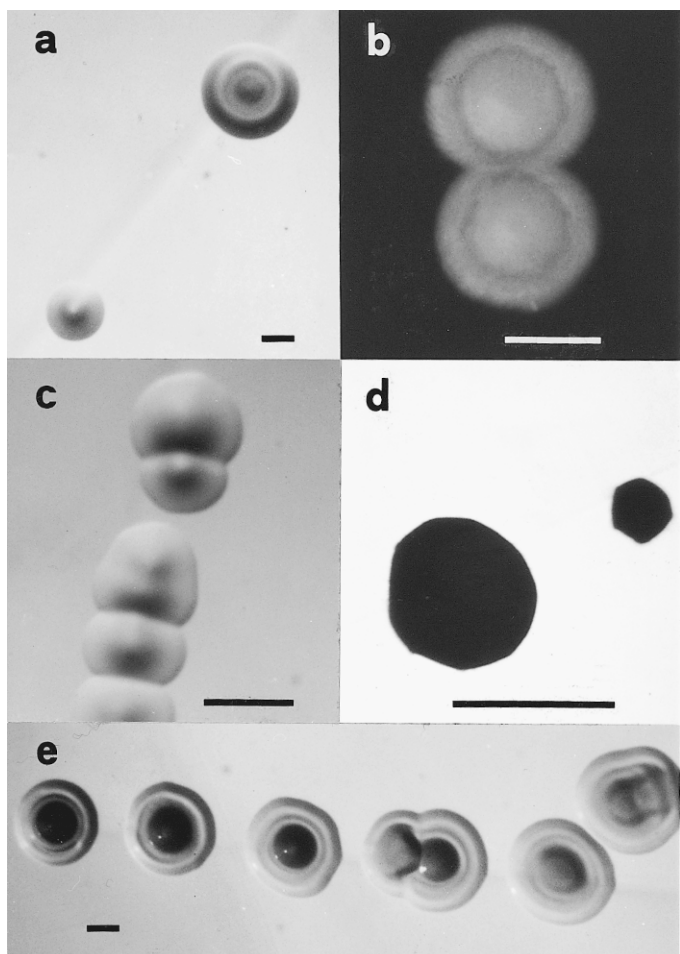


FIGURE BXII.α.132. Morphology of *Chelatobacter heintzii* colonies. (a), PCA-grown strain TE 8. (b), NTA-grown strain TE 8. (c), PCA-grown strain TE 9. (d), colony of NTA-grown strain TE 9 after 7 days, note the polygonal shape of the colonies. (e), colonies of PCA-grown strain TE 4. Bars = 0.5 mm.

For isolation and growth on agar plates this medium is supplemented with 1.5% agar plus NTA (0.5 g/l). For isolation, and in early experiments, vitamins were included in the medium, but later it was found that for growth of all strains of *Chelatobacter heintzii*, vitamins can be omitted.

Growth characteristics Isolates differed with respect to their growth on media containing high nutrient concentrations. Most of the strains grew poorly and only after heavy inoculation on PCA plates of the original concentration. However, faster growth occurred on diluted (10–20% of the original concentration) PCA

medium broth amended with 1.5% agar, although the colonies obtained were smaller.

Growth in batch culture with NTA as the only carbon/energy and nitrogen source results in the excretion of ammonia, causing an increase in pH of the culture medium. Under such conditions growth ceases at approximately pH 9 and, after transfer into new medium, the cells exhibit extended lag phases (often 5–10 d) until growth resumes. Growth with mixtures of utilizable carbon sources plus NTA resulted in a simultaneous utilization of the two substrates and in faster growth than with NTA alone. For example, during simultaneous utilization of glucose plus NTA, a maximum specific growth rate of 0.17 h^{-1} was reported for strain TE 8 compared to 0.11 h^{-1} during growth with NTA alone (Table BXII.α.115).

Nutritional and physiological characteristics The nutritional and physiological characteristics of different strains of *Chelatobacter heintzii* are listed in Table BXII.α.116. All strains utilized acetate, benzoate, citrate, glycollate, lactate, malate, propionate, pyruvate, succinate, *n*-butanol, ethanol, glycerol, *iso*-propanol, *n*-propanol; the sugars *N*-acetylglucosamine, arabinose, *D*-cellobiose, esculin, *D*-fructose, galactose, gentiobiose, *D*-glucose, inositol, *D*-maltose, mannitol, *D*-mannose, rhamnose, *D*-ribose, *D*-sorbitol, sucrose, *D*-xylose, *L*-alanine, *L*-arginine, *L*-aspartate, *L*-glutamate, *L*-glycine, *L*-lysine, *L*-phenylalanine, *L*-proline, *L*-serine, and acetamide. No strain grew with: adonitol, aniline, arabit, dimethyl formamide, dimethyl sulfoxide, erythritol, [S,S]-ethylene diamine disuccinate, formate, gluconate, *N*-(2-acetamido)-iminodiacetate, *D*-lactose, malonate, methane, methanol, methyl acetamide, methyl diethyl amine, *n*-decane, oxalate, phenol, tetraethyl ammonium chloride, triethanol amine, tris-hydroxymethyl amino methane, urea, xylene, and H_2/CO_2 .

In addition, none of the strains produced gas from glucose, indole or H_2S , was able to ferment sugars, to hydrolyze DNA, to fix N_2 , to denitrify with NTA or with glucose, to produce acid from glucose or ethanol, to grow on mannitol (*Rhizobium*) agar, or was acid fast. For all strains, growth was slightly affected by the presence of 1% NaCl and no growth occurred with 10% NaCl.

Metabolism and metabolic pathways A key nutritional feature of members of the genus *Chelatobacter* is their ability to grow with the chelating agent NTA as the only source of carbon, energy and nitrogen. The biochemistry of NTA utilization has been studied mainly in *Chelatobacter heintzii* ATCC 29600 (Egli, 1994) and is shown in Fig. BXII.α.133. After transport into the cell, two enzymatic steps are sufficient to transform NTA into the central metabolites glycine and glyoxylate. Transport of NTA is most probably energy-dependent (summarized in Bucheli-Witschel and Egli, 2001). Recently, it was suggested that free NTA (and not a metal-NTA complex) is the form that is transported (Bolton et al., 1996). This implies that the equilibrium between free NTA and metal-complexed NTA will be crucial for the transport of

TABLE BXII.α.115. Maximum specific growth rate of three NTA-utilizing strains during batch growth with various substrate combinations^{a,b}

Substrate	<i>Chelatobacter heintzii</i> TE 8	<i>Chelatobacter heintzii</i> TE 9	<i>Chelatococcus asaccharovorans</i> TE 1
NTA	0.11	0.1	0.07
NTA/acetate	0.15	0.13	0.13
NTA/glucose	0.17	0.13	0.07
NH_4^+ /acetate	0.19	0.23	0.12

^aAdapted from Egli et al. (1988).

^bGrowth rates (h^{-1}) measured at 30°C, pH 6.8–7.0, and substrate concentrations of 250 mg/l.

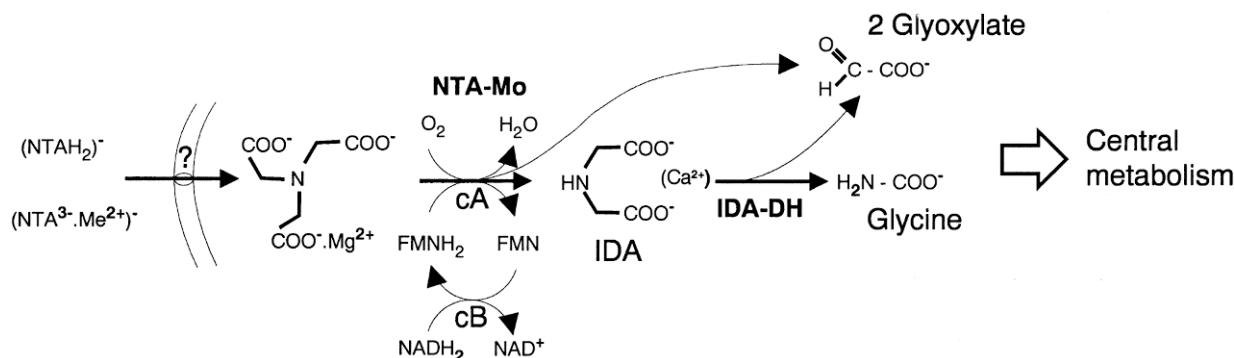


FIGURE BXII.α.133. Metabolic pathway for NTA in *Chelatobacter heintzii* ATCC 29600. IDA, membrane-bound iminodiacetate dehydrogenase; NTA-Mo, NTA monooxygenase, component A (cA) and B (cB), respectively. Note that the function of cB can be taken over by other FMN-reducing oxidoreductases.

this compound into the cell and that labile metal-NTA complexes should be degraded preferentially.

NTA monooxygenase The first intracellular step is catalyzed by a monooxygenase (NTA-Mo). NTA-Mo was originally isolated as a two component system, consisting of component cA and a FMN-containing protein cB with both components being present as dimers (Uetz et al., 1992). Immunological studies indicated that both cA and cB are present in all *Chelatobacter heintzii* strains tested so far (Table BXII.α.116; Uetz, 1992). It was demonstrated recently that cA acts as an independent monooxygenase whereas cB is supplying the enzyme with FMNH₂ and that FMN oxidoreductases from distantly related organisms and enzyme systems can supply cA with FMNH₂ (Witschel et al., 1997). The substrate specificity of NTA-Mo is very narrow, so far NTA complexed with Mg²⁺ is the only compound that is known to be accepted (Uetz et al., 1992). Neither free NTA nor Ca²⁺-complexed NTA is accepted as a substrate. Both components have been cloned and sequenced from *Chelatobacter heintzii* ATCC 29600 (see below).

Iminodiacetate dehydrogenase A membrane-bound iminodiacetate dehydrogenase (IDA-DH) which is present in all presently known NTA-degrading strains, was found to be the second enzyme in the catabolism of NTA (Uetz and Egli, 1993). The enzyme differs from succinate dehydrogenase and experiments with IDA-DH extracted from membranes and reconstituted in artificial membrane vesicles indicated that it feeds electrons abstracted from IDA into the electron transfer chain at the level of ubiquinone.

Regulation of NTA-degrading enzymes Recently, regulation of the ability to degrade NTA and the expression of NTA-Mo was studied under different growth conditions in carbon-limited continuous culture (Bally, 1994; Bally et al., 1994; Egli, 1995; Bally and Egli, 1996). NTA was consumed simultaneously with easily degradable carbon substrates such as, e.g., glucose and the extent of expression of NTA-Mo was dependent on the ratio of NTA:glucose in the feed medium (and, hence, the ratio of the two carbon sources consumed by the cell). Significant expression of NTA-Mo was only observed when the contribution of NTA to the total carbon consumed by the culture was higher than 1%. The experiments indicate that in wastewater treatment plants or the natural environment, NTA-degrading bacterial cells are probably not growing with NTA as a single carbon source but with mixtures of NTA plus naturally available carbon substrates, and that NTA-degrading enzymes are probably rarely fully induced under environmental growth conditions. These results are sup-

ported by studies from real and model wastewater treatment plants (Bally, 1994) showing that in plants receiving more than 1% of their organic carbon as NTA, both components of NTA-Mo were expressed, whereas in plants receiving only little NTA it was not detectable. Nevertheless, in both systems the fraction of NTA-degrading cells (as assessed with cell surface antibodies) was similar, indicating that under such conditions probably the induction of the catabolic enzymes was more responsible for successful degradation of NTA than the enrichment of NTA-degrading bacterial cells (Egli, 1995).

Genetics With regard to catabolic genes, in *Chelatobacter heintzii* ATCC 29600 the genes for the components cA and cB of NTA-Mo (see above) have been cloned and sequenced recently (Knobel et al., 1996). Two open reading frames (most probably chromosomally encoded) oriented divergently with an intergenic region of 307 bp could be assigned to the two components. The deduced gene products of *ntaA* showed significant homology only to SoxA (involved in dibenzothiophene degradation) and SnaA (involved in pristamycin synthesis), whereas that of *ntaB* shared weak homology in one domain with other NADH:FMN oxidoreductases. Unfortunately, these homologies give no conclusive answer yet with respect to the possible evolutionary origin of NTA-Mo. Additionally, an open reading frame was found downstream of *ntaA* which shares considerable homology in the N-terminal region with the GntR class of bacterial regulator proteins and, therefore, may encode a regulatory protein involved in the regulation of *ntaA* and *ntaB* expression. This information was recently confirmed (Xu et al., 1997). Hybridization experiments with different gene probes from *Chelatobacter heintzii* ATCC 29600 indicate that *ntaA* is highly conserved in the different strains investigated so far (even in *Chelatococcus asaccharovorans*), whereas *ntaB* was not detected in all strains. This confirms the recent observation that probably any FMN-reducing oxidoreductase can supply FMNH to NTA-Mo, i.e., component A (Witschel et al., 1997; Xu et al., 1997).

Antigenic structure Polyclonal antibodies (α-Cb and α-Cc) have been raised against whole cells (El-Banna, 1989; Wilberg et al., 1993) and isolated cell walls (Bally, 1994) of NTA-utilizing *Chelatobacter* and *Chelatococcus* strains. In Ouchterlony double diffusion or indirect immunofluorescence tests, the antisera raised against the two central NTA-utilizing *Chelatobacter* strains, i.e., *Chelatobacter heintzii* strains TE 6 and ATCC 29600, did not cross-react with cell homogenates from both *Chelatococcus asaccharovorans* strains. Similarly, the antiserum raised against *Chelatococcus asaccharovorans* strain TE 2 did not cross-react with homogenates

TABLE BXII.α.116. Physiological properties of NTA-utilizing, obligately aerobic strains of *Chelatobacter heintzii* and *Chelatococcus asaccharovorans*^{a,b,c}

Characteristic	<i>Chelatobacter heintzii</i> ^d	<i>Chelatococcus asaccharovorans</i> ^d
<i>Hydrolysis of:</i>		
Gelatin ^{e,f}	d	+
ONPG ^{f,g}	d	+
Urea ^f	d	+
Starch	— ^h	—
Protein	— ⁱ	—
Tween 80	— ^j	—
<i>Production of:</i>		
Nitrite ^{e,f}	d	+
Acetoin ^{e,k}	+ ^l	—
Pigment on King's medium	—	—
<i>Presence of:</i>		
Oxidase ^{e,m}	+	+
Catalase ^f	+ ⁿ	+
NTA-monooxygenase cA ^o	+	+
NTA-monooxygenase cB ^o	+	—
PHB	+ ^p	+
<i>Growth characteristics:</i>		
41°C	—	+
4°C	— ^q	+
KCN	+ ^r	+
Vitamin requirement	—	+
Tellurite ^s	— ^t	—
<i>Carbon substrates used:</i>		
Methylamine	+ ^u	—
Dimethylamine	+ ^u	—
Trimethylamine	+ ^u	—
Butyrate	+ ^v	—
Fucose	+ ^w	—
Glycyl-glycine	d	—
Glyoxylate	+ ^x	+
Iminodiacetate	+ ^y	—
D-(+)-Raffinose	d	—
Sarcosine	d	—
Skim milk	— ^z	—
Xylitol	—	—

^aSymbols: +, positive result obtained with seven or more of the nine strains; (+), weakly positive result; —, negative result obtained with seven or more of the nine strains; d, three to six strains out of nine strains tested differed in their reaction (results of individual strains in Egli et al., 1988).

^bData from Egli et al. (1988) and Egli and Weilenmann (1989).

^cMany of the tests were performed with both NTA-grown cells and cells grown on PCA plates.

^d*Chelatobacter heintzii* strains TE 4–TE 10 and ATCC 27109 and ATCC 29600; *Chelatococcus asaccharovorans* strains TE 1 and TE 2. All strains were enriched in batch culture, except for *Chelatobacter heintzii* strain TE 4.

^eOnly cells grown on NTA-agar plates were positive; cells grown on PCA plates were negative.

^fTest performed with API-20B.

^gAs in e, except for strain ATCC 27109 that gave positive results on NTA as well as PCA plates.

^hTE 6 and ATCC 29600 were slightly positive.

ⁱATCC 29600 was positive, TE 4 and 9 were slightly positive.

^jATCC 29600 was positive.

^kPCA-grown cells of strain TE 5 were negative.

^lATCC 27109 was negative.

^mPCA-grown cells of strains TE 4–8 and 10 were weakly positive, those of strain TE 9 were negative.

ⁿCA grown cells of TE 4 and 9 were negative.

^oResults from Uetz (1992).

^pTE 10 and ATCC 27109 were slightly positive.

^qATCC 27109 was positive.

^rTE 4–8 were only slightly positive.

^s+, formation of black colonies; —, no growth.

^tTE 9 and ATCC 27109 were positive.

^uTE 4 and TE 10 were negative.

^vTE 10 and ATCC 29600 were negative.

^wTE 9 and 10, ATCC 27109, and ATCC 29600 were weakly positive.

^xTE 4 and 10 were negative.

^yTE 4 was negative.

^zATCC 29600 was positive; TE 4 and TE 9 were slightly positive.

of the two central strains of *Chelatobacter heintzii* (Wilberg et al., 1993).

Cross-reaction of α-Cb was also tested with a variety of bacteria that were expected to co-exist with members of *Chelatobacter* in the same habitat. No cross reaction was observed for *Agrobacterium tumefaciens*, *Alcaligenes faecalis*, *Azotobacter chroococcum*, *Bradyrhizobium japonicum*, *Comamonas acidovorans*, *Comamonas testosteroni*, *Escherichia coli* ML30, *Klebsiella pneumoniae*, NTA-utilizing strain TE 11 (denitrifying), *Paracoccus denitrificans*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhizobium leguminosarum*, *Rhizobium meliloti* and *Rhodobacter* sp. (Wilberg et al., 1993). A slight cross-reaction was observed with *Carbophilus carboxidus* DSM 1086, *Aminobacter aminovorans* NCIB 9039, *Chelatobacter heintzii* strains TE 4 to TE 10, and *Ralstonia eutropha*. Highly specific antibodies were prepared from outer membrane fractions of *Chelatobacter heintzii* ATCC 29600 using a depletion method (Bally, 1994). The only bacteria still exhibiting significant cross-reaction with the purified antibodies were *Rhizobium phaseoli* and *Carbophilus carboxidus*.

Antibiotics and drug resistance The susceptibility of NTA-utilizing *Chelatobacter heintzii* and *Chelatococcus asaccharovorans* strains towards a range of antibiotics was tested (Auling et al., 1993a) and a summary of the results is provided in Table BXII.α.117. All *Chelatobacter heintzii* strains were resistant to nalidixic acid and sensitive towards penicillin G, ampicillin, carbenicillin, deoxycycline, vancomycin, and novobiocin. It has not yet been determined whether this extreme sensitivity towards β-lactam antibiotics is a property that allows the differentiation of *Chelatobacter* spp. from close neighbors. However, the resistance of all strains towards nalidixic acid might be used to isolate such strains with a medium amended with this antibiotic.

TABLE BXII.α.117. Resistance of *Chelatobacter heintzii* and *Chelatococcus asaccharovorans* towards a range of different antibiotics^{a,b}

Antibiotics ^c	<i>Chelatobacter heintzii</i> ^d	<i>Chelatococcus asaccharovorans</i> ^e
<i>β-Lactams:</i>		
Penicillin G, 10U	S	S
Ampicillin, 10 µg	S	d
Carbenicillin, 100 µg	S	S
Cephaloridine, 30 µg	S	R
Cephalexin, 30 µg	d	R
<i>Aminoglycosides:</i>		
Streptomycin, 10 µg	d	R
Neomycin, 30 µg	S	S
Kanamycin, 30 µg	d	S
Gentamycin, 10 µg	S	S
Tobramycin, 10 µg	d	S
<i>Other antibiotics:</i>		
Tetracycline, 30 µg	S	S
Doxycycline, 30 µg	S	S
Erythromycin, 15 µg	S	S
Chloramphenicol, 30 µg	d	d
Rifampicin, 30 µg	S	S
Polymyxin B, 300 U	d	S
Novobiocin, 30 µg	S	S
Vancomycin, 30 µg	S	R
Nalidixic acid, 30 µg	R	R
Trimethoprim, 1.25 µg; + sulfamethoxazole, 23.75 µg	d	R

^aSymbols: S, at least eight or all strains sensitive; R, at least eight or all strains resistant; d, strains differed in their reaction.

^bData from Auling et al. (1993a).

^cAbsolute amount of antibiotic used in test.

^dNine *Chelatobacter heintzii* strains were tested (TE 4 to 10, ATCC 27109, ATCC 29600).

^eTwo *Chelatococcus asaccharovorans* strain were tested (TE 1 and TE 2).

Ecology Data collected so far with surface antibodies (Wilberg et al., 1993; Bally, 1994) and 16S rRNA probes (Neef, 1997) indicate that members of the genus *Chelatobacter* are ubiquitously distributed in the environment. This is supported by the ready isolation of *Chelatobacter* strains from soil, sediment, surface and wastewater. Even in an environment with little chance of having ever been exposed to NTA, i.e., a small pristine alpine stream that contained no detectable NTA ($<0.2 \mu\text{g/l}$), cells cross-reacting with antibodies raised against *Chelatobacter heintzii* ATCC 29600 were detected at 1.2×10^5 cells per liter (corresponding to 0.27% of the total bacterial population) (Bally, 1994). In nutrient-rich environments such as eutrophic surface waters or wastewater treatment plants, the total number of cross-reacting cells generally increased by several orders of magnitude. For example, both Wilberg et al. (1993) and Bally (1994) reported numbers of NTA-degrading *Chelatobacter* cells in aerated tanks of several Swiss wastewater treatment plants in the range of 10^{10} cells/l (corresponding to 0.1–1% of the total bacterial population). Similar figures were recently reported using a molecular probe specific for the 16S rRNA of *Chelatobacter heintzii* ATCC 29600 (Neef, 1997).

Polyamines and ubiquinones All *Chelatobacter heintzii* strains contained *sym*-homospermidine as the main, putrescine and spermidine as major, and spermine as minor polyamine compounds (Auling et al., 1993a). Ubiquinone Q-10 was present in all strains. As it has been frequently stated that NTA is utilized by specialized pseudomonads, this polyamine and quinone profile allows *Chelatobacter heintzii* to be clearly distinguished from the true (fluorescent) *Pseudomonas* spp., which contain putrescine and spermidine as the main polyamines and ubiquinone Q-9 (Auling et al., 1991, 1993a).

Soluble protein patterns The soluble protein pattern for *Chelatobacter heintzii* has been compared to that of *Chelatococcus asacharovorans* (Auling et al., 1993a). *Chelatobacter* and *Chelatococcus* strains exhibited clearly different patterns. Among the different *Chelatobacter* strains, the protein patterns were highly similar, except for two isolates (strains TE 4 and 7). Although the soluble protein patterns of strains TE 4 and 7 were virtually identical, the two strains differed considerably with respect to their nutritional and physiological properties (Egli et al., 1988).

Miscellaneous comments In the 1970s, eutrophication of surface waters by phosphorus from detergents and agriculture stimulated discussion about banning sodium tripolyphosphate (STPP) from laundry detergents and replacing it with non-phosphorus-containing metal-chelating agents such as aminopolycarboxylic acids (Mottola, 1974; Tiedje, 1980; Anderson et al., 1985). Aminopolycarboxylic acids are metal-sequestering compounds used in large amounts in a variety of domestic and industrial applications including cleaning agents, agricultural fertilizers, for water treatment and descaling of boilers, in the photographic industry, the dyeing of textiles, during pulp and paper production, for metal finishing and rubber processing, or in food, pharmaceuticals, and cosmetics (McCrary and Howard, 1979; Egli, 1988; Egli et al., 1990). Aminopolycarboxylic acids include both man-made (e.g., ethylenediaminetetraacetic acid [EDTA] and nitrilotriacetic acid [NTA]) and microbially synthesized (e.g., *S,S*-ethylenediaminedisuccinic acid) compounds. The most extensively used synthetic chelating agents are EDTA and NTA. All applications are water based and therefore their susceptibility to biodegradation during wastewater treatment and in the aquatic environment is an important criterion for assessing their environmental impact and toxicity (Anderson et al., 1985).

Of all the synthetic chelating agents, NTA and EDTA have received the most attention, NTA because of its controversial application in laundry detergents as a substitute for sodium tripolyphosphate, and EDTA because of its slow biodegradability and its ubiquitous presence in aqueous systems (Tiedje, 1980; Wolf and Gilbert, 1992).

It was soon established that elimination of NTA, one of the most promising substitutes for STPP, from wastewater and natural waters was exclusively brought about by microbial action. This was the motivation for setting up the first enrichment cultures to isolate NTA-degrading microorganisms (Focht and Joseph, 1971; Cripps and Noble, 1973; Enfors and Molin, 1973a, b; Tiedje et al., 1973; Kakii et al., 1986). Most of these isolates were identified based on rather superficial characterization and assigned to the genus *Pseudomonas*, although the isolation of NTA-utilizing nonpseudomonads was also reported (Egli et al., 1990). However, with the exception of the two strains deposited at the ATCC (strains ATCC 29600 and 27109), none of these isolates seems to be available anymore. Despite the poor characterization of these isolates, the (probably false) conclusion was drawn that the biodegradation of NTA was primarily a trait inherent to specialized *Pseudomonas* strains (Tiedje, 1980; Anderson et al., 1985). From the description given in the original publications and the fact that the two ATCC strains were described as similar, it seems highly likely that most of these isolates were the first *Chelatobacter* strains isolated from the environment. Today the ubiquitous occurrence of members of the genus *Chelatobacter* and *Chelatococcus* (Wilberg et al., 1993; Bally, 1994) gives a strong indication that these bacteria are a major component of the NTA-degrading microbial community in wastewater treatment plants and aerobic environments.

Other strains reported to grow at the expense of NTA have included *Bacillus*, *Listeria*, *Rhodococcus*, and yeast species (Egli, 1994). However, most of these strains have been poorly characterized and were lost. Nevertheless, these reports suggest that the ability to utilize NTA is probably not restricted to *Chelatobacter* and *Chelatococcus* species. This is supported by the recent finding of an EDTA-degrading isolate (clearly different from *Chelatobacter* and *Chelatococcus*) that was also able to metabolize NTA (Witschel et al., 1997).

ENRICHMENT AND ISOLATION PROCEDURES

From soil, wastewater, or surface waters, strains belonging to the genus *Chelatobacter* can be easily enriched in either batch or chemostat cultures using NTA as the only source of carbon, energy, and nitrogen (Egli et al., 1988; Egli and Weilenmann, 1989). All of the presently known strains of *Chelatobacter* are resistant towards nalidixic acid (Table BXII.α.117). This might be used to isolate such strains with a medium containing this antibiotic.

MAINTENANCE PROCEDURES

All strains can be maintained in a freeze-dried condition. Alternatively, cultures grown in SM with NTA and amended with either glycerol (15%, v/v) or DMSO (50%, v/v) can be stored in liquid nitrogen. However, with either method revival of cultures may take a while. Best results have been obtained when streaking cultures from liquid nitrogen directly onto agar plates with SM containing NTA as the only carbon and nitrogen source. When maintaining cultures on selective NTA agar plates, it is recommended to transfer them to new plates at least every week because growth leads to an increased pH in and around the colonies. Cells stored under such conditions exhibit long lag phases (up to several weeks) before they restart growing. Liquid cultures

behave similarly. Long lag times and irreproducible growth can be avoided by transferring the cells to fresh medium whilst they are still growing exponentially. Note that concentrations of NTA that are too high, combined with low buffering capacity of the medium or no pH control, may lead to an early cessation of growth.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Growth with NTA as the only source of carbon, energy, and nitrogen is a key property of *Chelatobacter*. Consumption of NTA can be measured either by the disappearance of dissolved organic carbon (DOC) paralleled by the excretion of ammonia, or via the consumption of NTA. A HPLC method specially developed for the analysis of NTA in culture media and cell extracts has been described by Schneider et al. (1988).

DIFFERENTIATION OF THE GENUS *CHELATOBACTER* FROM OTHER GENERA

The presently available information indicates that members of the genus *Chelatobacter* are most likely closely related to members of the genus *Aminobacter*. The nutritional and physiological properties that allow separation of the two genera are listed in Table BXII.α.118.

TAXONOMIC COMMENTS

The presence of ubiquinone Q-10 (Egli et al., 1988), and the characteristic polyamine pattern with *sym*-homospermidine as the main, and putrescine and spermidine as major components (Auling et al., 1993a), clearly allocates members of the genus *Chelatobacter* to the *Alphaproteobacteria*, and to the family *Rhizobiaceae* (D. Kuykendall, personal communication). The polyamine pattern indicates a relationship to *Phyllobacterium* (Auling et al., 1991) but distinguishes *Chelatobacter* from *Ochrobactrum*. A weak serological cross-reaction with *Aminobacter aminovorans* (Green and Gillis, 1989; Urakami et al., 1992) and even less with *Carpophilus carboxidus* (Meyer et al., 1993), formerly "*Alcaligenes carboxydus*" (Auling et al., 1988), was observed (Wilberg et al., 1993). Oligonucleotide catalogs indicated the latter as the nearest neighbor of *Chelatobacter* (Auling et al., 1993a). Meanwhile, 16S rDNA sequence data allow grouping of *Chelatobacter* with *Mesorhizobium* (Jarvis et al., 1997), 4-chloro-2-methylphenol-degrading (Lechner et al., 1995) members of *Defluviobacter* (Fritsche et al., 1999a), two species of *Pseudaminobacter* (Katayama-Fujimura et al., 1983, 1984b; Kämpfer et al., 1999), and *Aminobacter* (Urakami et al., 1992).

The genus presently comprises nine strains and contains only

TABLE BXII.α.118. Nutritional and physiological properties that allow differentiation between the genera *Chelatobacter* and *Aminobacter*^{a,b}

Characteristics	<i>Chelatobacter</i>	<i>Aminobacter aminovorans</i> ^c	<i>A. aganoensis</i> , <i>A. niigataensis</i> ^d
Morphology	many pleomorphic cells	rods, pleomorphic forms	rods, budding, no pleomorphic forms reported
<i>Substrate utilization:</i>			
NTA ^e	+	—	—
Aspartate	+	—	+
Citrate	+ ^f	—	—
Dimethylamine	+ ^g	—	+
Ethanol	+	—	—
Malate	+ ^f	—	—/+
Phenylalanine	+	—	—
Fucose	—	d	nd
Sarcosine	d ^f	—	nd
Succinate	+	— ^c (+) ^d	+
Glutamate	+	d	nd
Growth on mannitol (<i>Rhizobium</i>) agar	—	+	+
Growth on standard complex medium ^h	weak	abundant	weak
Growth at 4°C	—	nd	nd
Growth at 5°C	nd	+	nd
Urease	— ⁱ	+ (—) ^d	—
Nitrate to nitrite	— ^j	d	nd
Gelatin liquification	+ ^k	—	—
Acid from arabinose	+	+	—
Voges-Proskauer	+ ^l	—	—
16S rRNA gene sequence to <i>C. heintzii</i> DSM6450 Tm	100%	99.8%	99.9%
Serological relationship (cross reaction with ATCC 29600)	+	—	nd

^aData collected from Egli et al. (1988), Egli and Weilenmann (1989), Green and Gillis (1989), and Urakami et al. (1992).

^bSymbols: +, positive; —, negative; d, individual strains differ in their properties; nd, not reported.

^cMost data from Green and Gillis (1989) (including the strains NCIB 9039, 11590, and 11591, later assigned to this species by Urakami et al., 1992).

^dData from Urakami et al. (1992).

^eStrains tested in our laboratory were *A. aminovorans* NCIB 9039, *A. aganoensis* DSM 7051, *A. niigataensis* DSM 7050.

^fFrom Egli et al. (1988), differing from Kämpfer et al. (1999).

^gTE 4 and 10 are negative.

^hPlate count agar, nutrient broth, peptone water, or PYG broth.

ⁱTE 5 and 9, ATCC 27109, and 29600 are positive.

^jTE 7 and 8 are positive, TE 4 and 5 slightly positive.

^kTE 9 and 10 and ATCC 29600 are negative.

^lATCC 27109 negative.

^mFrom Kämpfer et al. (1999). Strains used were *Chelatobacter heintzii* DSM 6450^T, *Aminobacter aminovorans* DSM 7048^T, *A. aganoensis* DSM 7051^T, and *A. niigataensis* DSM 7050^T.

a single species, *Chelatobacter heintzii*. Based on DNA–DNA hybridization studies (Auling et al., 1993a), the genus is divided into two groups, A1 and A2 (Fig. BXII.α.134). However, this division does not reflect the nutritional and physiological properties of the different *Chelatobacter* isolates (see Table BXII.α.116). The reference strain for DNA homology cluster A1 is *C. heintzii* strain ATCC 29600, which is also the type strain of the species. Strain *C. heintzii* TE 6 is the central strain of the DNA homology cluster A2.

The increasing availability of new relatives of *Chelatobacter* may require a change in its nomenclatural status in the future; a clustering of *Chelatobacter* with *Aminobacter* was recently proposed by Kämpfer et al. (2002). However, any revision based on phylogenetic considerations will meet with problems, since the related taxa are located within the notoriously “shallow” branch formerly known as the “alpha-2” branch of the *Proteobacteria*.

ACKNOWLEDGMENTS

We are indebted to Ernst Wehrli for supplying us with excellent electron micrographs. In addition, we thank D. Kuykendall for allowing us to include unpublished information. TE would like to thank H.U. Weilenmann and all the students that have contributed to the study of NTA-utilizing microorganisms. Furthermore, the generous financial support of research on NTA in the laboratory of TE by grants from the Swiss National Science Foundation, Lever AG Switzerland and Unilever Port Sunlight, the Research Commission of ETH Zürich, and by EAWAG is gratefully acknowledged.

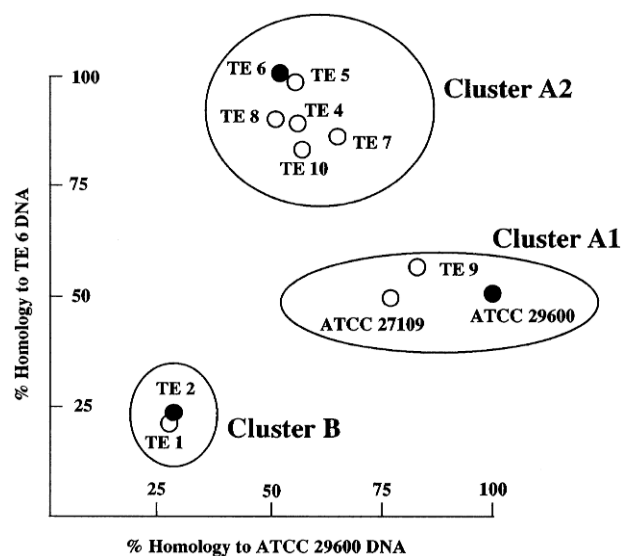


FIGURE BXII.α.134. DNA similarity groups of Gram-negative, obligately aerobic, NTA-utilizing isolates belonging to the genera *Chelatobacter* and *Chelatococcus* (Reproduced with permission from G. Auling et al., *Systematic and Applied Microbiology* 16: 104–112, 1993, ©Urban & Fischer Verlag, Jena.)

List of species of the genus *Chelatobacter*

- Chelatobacter heintzii*** Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624^{VP} (Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 110.) *heintzii* i.i. M.L. gen. n. *heintzii* of Heintz; named after the chemist W. Heintz who was the first to synthesize the chelating agent NTA (Heintz 1862, 1865) and to describe some of its properties.

The characteristics are as described for the genus and listed in Tables BXII.α.115, BXII.α.116, and BXII.α.117.

The mol% G + C of the DNA is: 62–63 (T_m).

Type strain: ATCC 29600, DSM 10368.

Additional Remarks: All NTA-utilizing *Chelatobacter heintzii* strains described here have been deposited at the German Collection of Microorganisms and Cell Cultures (DSM), Braunschweig, Germany, where they have been assigned the following numbers: TE 4, DSM 6463; TE 5, DSM 6464; TE 6, DSM 6465; TE 7, DSM 6466; TE 8, DSM 6449; TE 9, DSM 6450; TE 10, DSM 6451. The other strains are available from the American Type Culture Collection, Rockville, MD, U.S.A., i.e., the strain isolated by Tiedje et al. (1973), namely *Chelatobacter heintzii* ATCC 29600 (also available from DSM under accession number DSM 10368) and ATCC 27109 (originally isolated by Focht and Joseph, 1971).

Genus VI. *Ensifer* Casida 1982, 343^{VP}

DAVID L. BALKWILL

En'si.fer. L. adj. *ensifer* sword-bearing; M.L. masc. *Ensifer* sword bearer.

Rods 0.7–1.1 × 1.0–1.9 μm, occurring singly or in pairs. **Reproduction by budding at one pole of the cell**, with the bud then elongating to produce **asymmetric polar growth**. **Attaches endwise to various living Gram-positive and Gram-negative host bacteria and may cause lysis of the host cells**. **Host cells are not required for growth**. Gram negative, but may stain poorly. **Motile by a tuft of three to five subpolar flagella**. Aerobic; does not grow anaerobically in the presence of light. Optimal growth occurs at 27°C; good growth occurs at 20°C and 37°C. Not heat resistant. Weakly catalase positive. Nitrate and nitrite are reduced. Nitrification is negative for nitrite and ammonia. The **metabolism of glucose and galactose is oxidative**. Growth is inhibited by 4% NaCl but not by 2.5% NaCl. Grows well on most media. Definite but slow growth on soil extract agar and on 1.5% Noble agar in distilled water. Agar is not hydrolyzed. Utilizes a variety of organic carbon sources. A segment of the *Ensifer* 16S rDNA sequence (TACGGAGACGTTT, corresponding to positions 1009 through

1021 in the *Escherichia coli* 16S rDNA sequence; Brosius et al., 1978) might represent a signature sequence for this genus, as it did not match any other bacterial sequences available in public databases as of June, 2003.

The mol% G + C of the DNA is: 67 (T_m) and 63 (Bd).

Type species: *Ensifer adhaerens* Casida 1982, 343.

FURTHER DESCRIPTIVE INFORMATION

E. adhaerens was originally designated as strain A (Casida, 1980). Growth of *E. adhaerens* is initiated by budding at one pole of the cell, after which the bud elongates to produce asymmetric polar growth (Fig. BXII.α.135). This growth usually widens to equal the diameter of the mother cell. The daughter cell eventually separates from the mother cell by binary fission. Either the mother and daughter cells are the same size, or the daughter cell is smaller.

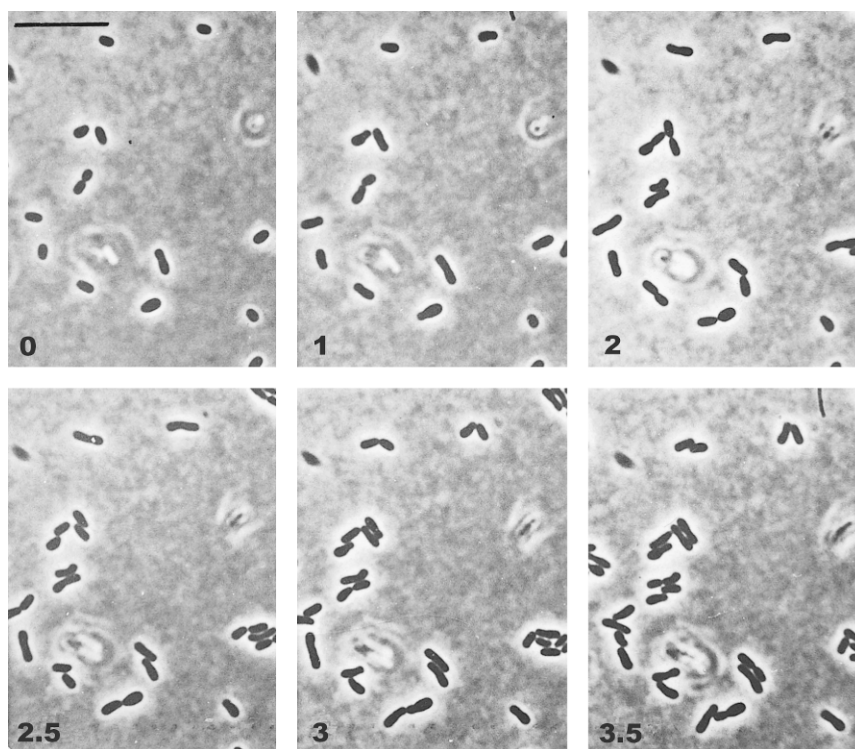


FIGURE BXII.α.135. Growth of *Ensifer adhaerens* on full-strength heart infusion agar. Incubation was for 0, 1, 2, 2.5, 3, and 3.5 h. Phase-contrast microscopy of a slide culture. Bar = 10 μ m. (Reproduced with permission from L.E. Casida, Jr., International Journal of Systematic Bacteriology 32: 339–345, 1982, ©International Union of Microbiological Societies.)

Measurements from electron micrographs indicate that some of the nonattached daughter cells are 20–30% smaller (length or width or both) than the nondividing mother cells. There is no tube or filament between mother and daughter cells during binary fission. After fission, growth resumes as new buds at the newly formed poles of both the mother and daughter cells. There does not appear to be any requirement for a rest or maturation period before growth resumes. Bud formation does not always occur at the pole of the cell; buds sometimes form on the side of a mother cell in old cultures.

The budding and division processes of *E. adhaerens* are similar to those of *Rhodopseudomonas blastica* (Eckersley and Dow, 1980). However, the latter organism is photosynthetic, grows anaerobically in the light, and divides only when the mother and daughter cells are of equal size. Moreover, the daughter cell must undergo an obligate period of maturation before it initiates growth.

A bar of darkly stained material is frequently visible along the side of the cell in whole-cell preparations of *E. adhaerens* that are stained with uranyl acetate and observed by electron microscopy. This bar becomes wider near one or both poles of the cell. When cells of *E. adhaerens* are attached to host cells, such as those of *Micrococcus luteus*, this bar often extends between the *E. adhaerens* cell and the host cell. The chemical composition of the bar is not known. In addition, it is not readily apparent in whole-cell preparations negatively stained with phosphotungstic acid instead of uranyl acetate, and it has not been located unequivocally in thin-sectioned material.

When *E. adhaerens* is grown in the presence of *M. luteus* (or some other sensitive bacterium) on agar medium, large numbers of *E. adhaerens* cells attach endwise to *M. luteus* cells. The *E. adhaerens* cells are situated side by side, closely packed, in a picket fence arrangement. When the prevailing nutrient and pH con-

ditions (in soil or on laboratory media; pH 6 is optimal) are satisfactory, this arrangement eventually results in lysis of the *M. luteus* cells. There is no specialized morphological structure on the *E. adhaerens* cell for attachment purposes. Rosettes are not formed.

E. adhaerens is not an obligate predator. It grows well on most media in the absence of potential host cells. In fact, the overall growth of *E. adhaerens* usually does not increase as a result of tracking (Casida, 1980) or lysis of *M. luteus* cells. However, some increased growth of *E. adhaerens* does occur when both organisms are placed on Noble agar with or without 0.1% glucose. Thus *E. adhaerens* probably benefits from this interaction only if the nutritive value of the medium is very low, a situation that is comparable to the presumed low availability of nutrients occurring in soils that have not recently received organic matter. In soil, *E. adhaerens* cells attach directly to host cells, so that even the small amounts of lytic factor produced under low-nutrient conditions can be used effectively in lysing host cells. On agar media, this lytic factor is diffusible and can act on host cells at a distance from the *E. adhaerens* cells.

E. adhaerens produces colonies 10–15 mm in diameter after 6 d on heart infusion agar prepared at one-tenth of the recommended strength (but containing 1.5% agar). These colonies are grayish white, circular with undulate margins, convex, slimy, moist, and opaque (but may appear almost translucent because of excessive slime production). Growth on agar slants is abundant, opaque, grayish white, smooth, flat, slimy, and moist. Pellicle and sediment (with little turbidity) are produced in a broth medium containing 0.5% peptone, 0.1% yeast extract, and 0.1% glucose.

Definite but slow growth occurs during sequential transfers on 1.5% Noble agar in distilled water. Host cells are not required,

and the agar is not hydrolyzed. This growth is equivalent to that obtained on soil extract agar. Good growth is obtained on a synthetic medium containing 0.1% glucose, 0.1% NH_4NO_3 , 0.1% KH_2PO_4 , 0.1% NaCl, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% L-glutamic acid, and 1.5% agar (pH 7.0). *E. adhaerens* utilizes a variety of organic carbon sources including glucose, galactose, mannose, rhamnose, xylose, mannitol, sorbitol, glycerol, L-glutamic acid, L-alanine, L-asparagine, and L-glutamine. Acetate is used only slowly and does not inhibit glucose utilization. *E. adhaerens* grows on pure gelatin without hydrolysis. Starch is not hydrolyzed. Good growth occurs on blood agar, with no hemolysis. Good growth also occurs on desoxycholate agar, initially appearing whitish purple and then changing to buff.

The metabolism (Hugh and Leifson, 1953) of glucose and galactose is oxidative. No growth occurs under petrolatum. Gas is not produced in the absence of petrolatum; only trace amounts of acid, if any, are produced.

E. adhaerens cells do not survive 30 s of heating in tap water at 71°C.

Thirteen lytic phages for *E. adhaerens* were isolated from soil by Germida and Casida (1983) and used for phage typing of eight *E. adhaerens* host strains. The phages were also used in assays to monitor *E. adhaerens* predatory activity against other bacteria in soil (see below).

The habitat for *E. adhaerens* is soil. The numbers of *E. adhaerens* cells in soils can be estimated (Casida, 1980) by gently applying dilutions of soil to the surfaces of pregrown lawns of *M. luteus* cells (one-tenth-strength heart infusion agar, with 1.5% agar). After continued incubation, *E. adhaerens* produces small, thin, transparent colonies that appear as small moist areas that expand and coalesce. These colonies are not visible by transmitted light. The plates must be viewed from above, using light arriving at an oblique angle. The *E. adhaerens* colonies are on the surface of the *M. luteus* lawn. Under these conditions, they neither penetrate the lawn nor lyse the *M. luteus* cells. The cell numbers of specific *E. adhaerens* strains in soil can also be estimated with a most probable number (MPN) method that combines phage analysis with a dilution frequency procedure (Makkar and Casida, 1987b). A small (but known) number of lytic phage specific for the strain to be enumerated is added to each soil dilution. A ten-fold or greater increase in PFU after a suitable incubation period is then taken to indicate that *E. adhaerens* host cells were present in a dilution. This method is capable of detecting *E. adhaerens* strains in soils even when they are present in very low numbers.

E. adhaerens has been isolated from a range of soil types. However, the strains from different soils do not necessarily cross-react when tested by phage typing (Germida and Casida, 1983). The predatory activity of *E. adhaerens* and its host range can be detected and followed in soils with the indirect phage analysis procedure of Germida and Casida (1983). Some bacteria, such as a myxobacterium and a *Streptomyces* predatory bacterium, succumb to *E. adhaerens* attack in soil but do not do so in laboratory cultures.

Indigenous *E. adhaerens* cells in soil attack only certain species of bacteria that are added to the soil. Studies using indirect phage analysis (Germida and Casida, 1983; Zeph, 1986; Zeph and Casida, 1986) have shown that *E. adhaerens* attacks *Agromyces ramosus*, *Micrococcus luteus*, a *Myxococcus* species soil isolate, *Staphylococcus aureus*, and two *Streptomyces* soil strains (C2 and 34). *E. adhaerens* does not attack *Actinomyces humiferus*, *Agrobacterium tumefaciens*, *Arthrobacter globiformis*, *Azotobacter vinelandii*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum*, *Salmonella typhi*, or *Sinorhizobium meliloti*. However, in a few instances *E. adhaerens* will

attack another bacterial predator, such as the *Myxococcus* soil isolate that is itself attacking certain of the above bacteria (Germida and Casida, 1983).

E. adhaerens is a component of a sequence of three predatory bacteria in soil that respond naturally when host cells, such as those of *M. luteus*, are added to the soil (Casida, 1980). A *Streptomyces* predator responds quickly with growth and attack on *M. luteus*. This is followed by growth of *E. adhaerens*, which attaches to and lyses both *M. luteus* and the streptomycete. Finally, a myxobacterium (*Myxococcus*) multiplies and attacks residual *M. luteus* cells but is, in turn, attacked by *E. adhaerens*. *E. coli* is not attacked directly by *E. adhaerens* in soil. Instead, the *E. coli* cells activate germination of the myxobacterium microcysts so that *E. coli* is destroyed, and this is followed by *E. adhaerens* attack on the myxobacterium (Germida and Casida, 1983; Liu and Casida, 1983).

E. adhaerens can also activate *Bacillus* spores (Mormak and Casida, 1985). In laboratory cultures, *E. adhaerens* cells promote a reversible germination of *B. subtilis* spores that does not proceed beyond the activation stage. Indigenous *E. adhaerens*-like bacteria promote the same type of limited spore germination in unamended soil. However, spores associated with *E. adhaerens*-like bacteria in soils that have been nutritionally enriched by incubation with ground alfalfa progress further in the germination process, and some of them may germinate fully, as implied by the eventual appearance of ghosted vegetative cells. There is no direct evidence that the indigenous *E. adhaerens*-like bacteria attack the germinated spores in nutritionally enriched soil, and cells of *E. adhaerens* do not attack vegetative cells of *B. subtilis* in laboratory cultures. Mormak and Casida (1985) have suggested that because small quantities of dipicolinic acid, calcium, and certain amino acids are released during spore activation, *E. adhaerens* and other soil bacteria may utilize these compounds for small amounts of growth or for maintaining cell viability.

E. adhaerens attaches to other bacteria in soil, but it also attaches to other bacteria (or does not become detached) during the preparation of dilutions of soil for plating of other soil organisms. Therefore, it may be in the colonies of the other soil organisms, and it may not be apparent that this is the case. *E. adhaerens* is difficult to remove from cultures of other bacteria unless the other organisms will grow on media containing 4% NaCl. *E. adhaerens* does not grow in the presence of 4% NaCl.

Germida et al. (1985) have reported that *E. adhaerens* can be used to bioassay for plant-available manganese in soils. The assay is specific for manganese, and the results correlate significantly with plant yields.

ENRICHMENT AND ISOLATION PROCEDURES

Variations of two procedures can be used for enrichment and isolation of *E. adhaerens*. In the first procedure, washed *M. luteus* cells, or cells of some other host organism, are added to natural soil, followed by the addition of enough water to adjust the soil water content to 50–60% of the soil's moisture-holding capacity. Alternatively, the cells are mixed with soil and sand in a soil percolation device (Germida and Casida, 1983), and water is percolated through the column of soil. Incubation is for 4–6 days at 25–27°C. After incubation, dilutions of soil are plated on desoxycholate agar or MacConkey agar. The dilutions of soil may also be spread carefully over the surfaces of pregrown lawns of *M. luteus* (on one-tenth strength heart infusion agar, with 1.5% agar), after which the *M. luteus* plates are incubated further. (See previous description for the appearance of the resulting *E. adhaerens* colonies.)

In the second isolation procedure, *M. luteus* cells, or cells of

some other host organism, are applied as a smear to a sterile glass slide. The cells are allowed to become just dry at room temperature. They are then immediately placed in contact with natural soil. The slides can be partially buried in soil, either outdoors or in the laboratory. They are placed vertically in the soil, so that only the top 2 cm (approximately) protrudes. Water is added to the soil at intervals to maintain a range of 40–65% of moisture-holding capacity. Alternatively, a sterile glass ring 11 mm high \times 25 mm in diameter (e.g., as cut from a test tube) may be placed on the smear and filled with soil. The soil is tamped lightly to ensure direct contact with the smear. Moisture-holding capacity is adjusted to 65%, and incubation is at 27°C in sterile Petri plates. Additional water is added as needed. The incubation time for the *M. luteus* host cells is 3–4 d. After completion of the incubation of these slides, or of buried slides, the soil is gently removed to expose the area of the slide surface where the *M. luteus* smear was placed. An inoculating loop that has been heated and plunged into agar while still hot is touched against this area of the slide. The loop is then streaked through a lawn of *M. luteus* cells or onto the surfaces of plates of desoxycholate or MacConkey agar without bacterial lawns.

MAINTENANCE PROCEDURES

E. adhaerens survives well on refrigerated slants of heart infusion agar made up at one-tenth strength (with 1.5% agar). It can also be lyophilized by common procedures used for aerobes.

DIFFERENTIATION OF THE GENUS *ENSIFER* FROM OTHER GENERA

The genus *Ensifer* is separated from other genera of aerobic, motile, nonphotosynthetic, Gram-negative rods by its method of multiplication. It reproduces by budding at one pole of the cell, with the bud then elongating to produce asymmetric polar growth. Separation of the cells occurs by binary fission, after which growth resumes immediately as new buds at the newly formed poles of both the mother and daughter cells.

In addition to the above, *E. adhaerens* attaches with one of its poles to various species of Gram-positive and Gram-negative bacteria. If enough *E. adhaerens* cells are present, they will position themselves side by side in a picket fence arrangement around the host bacterium. *M. luteus* is a good host organism for demonstrating this behavior. Depending on the species of the host involved in the interaction, *E. adhaerens* may proceed to kill and lyse the host if the pH and background nutritional level are suitable.

E. adhaerens can be differentiated, or even removed, from some other genera of bacteria by using a medium containing 4% NaCl. *E. adhaerens* does not grow in the presence of 4% NaCl but can grow in the presence of 2% NaCl.

The 16S rDNA sequence for *E. adhaerens* differs from those of all species of *Sinorhizobium* (the most closely related genus according to phylogenetic analyses) at positions 658, 659/746, 747, and 1012/1017, and from those of all but one species of *Sinorhizobium* at positions 1010/1019 and 1011/1018 (see below for details). The segment of the *E. adhaerens* 16S rDNA sequence from positions 1009 through 1021 did not match any bacterial sequences in public databases (as of June, 2003) and, therefore, may be unique to *Ensifer*.

TAXONOMIC COMMENTS

E. adhaerens-like bacteria have been isolated from several soils. These bacteria have the characteristics of *E. adhaerens*, but do not demonstrate a cross-reaction with strains A or 7A when they

are examined by phage typing. These *E. adhaerens*-like bacteria are considered to be strains of *E. adhaerens*, with the lack of cross-reaction being due to strain specificity of the particular bacteriophages that have been used.

16S rDNA sequences have been determined for two strains of *E. adhaerens*: ATCC 33212^T and ATCC 33499 (Balkwill, unpublished data; GenBank accession numbers AF191739 and AF191738, respectively). Phylogenetic analyses of these sequences with distance matrix (Fig. BXII.α.136), parsimony, and maximum likelihood methods indicated that *E. adhaerens* should be placed in the *Rhizobiaceae*. Within this family, *E. adhaerens* is most closely related to the genus *Sinorhizobium*. Similarities between the sequences for *E. adhaerens* and those for validly published species of *Sinorhizobium* are high (98.2% to 99.0%). On the other hand, the *Sinorhizobium* and *Ensifer* strains are always assigned to distinct (but closely related) clusters within the phylogenetic tree (see Fig. BXII.α.136), and the node at which the branching between these two clusters occurs is resolved consistently regardless of the method used to analyze the sequences. A number of consistent differences between the *Ensifer* and all available *Sinorhizobium* 16S rDNA sequences were also noted. These were located at *E. coli* positions (Brosius et al., 1978) 658

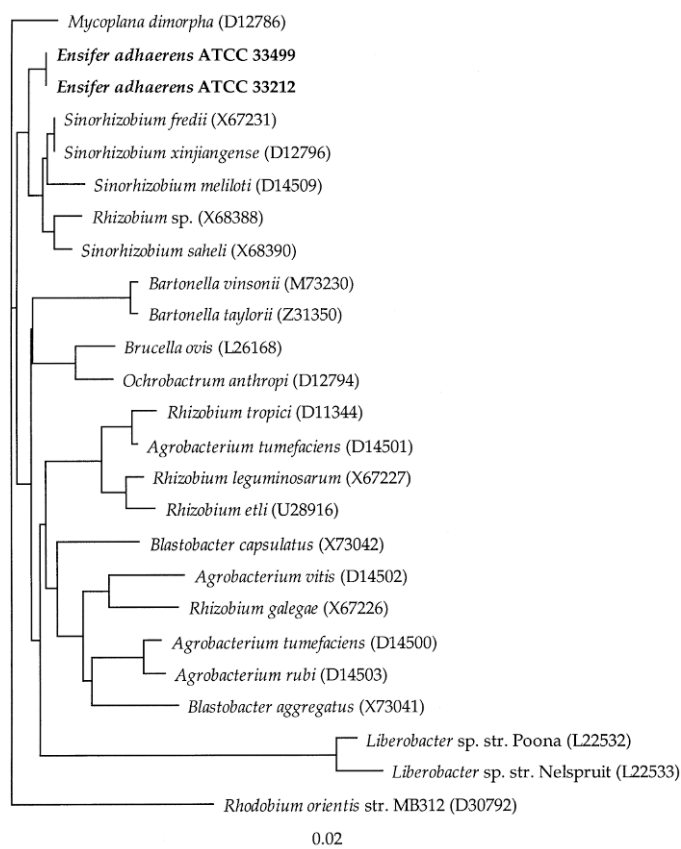


FIGURE BXII.α.136. Phylogenetic tree for two strains of *Ensifer adhaerens* and selected strains of *Bacteria*, based on a distance matrix analysis. Bar = 2 substitutions per 100 bases. *Rhodobium orientis* was used as the outgroup. Parsimony and maximum likelihood analyses yielded virtually identical trees with respect to the positions of *Ensifer* and *Sinorhizobium* and the branching patterns within the cluster containing strains of these two genera. The numbers in parentheses are the GenBank/EMBL accession numbers for the sequences used in the analysis. The GenBank accession numbers for the *Ensifer* sequences are as follows: *E. adhaerens* ATCC 33212^T, AF191739; *E. adhaerens* ATCC 33499, AF191738.

(A in *Ensifer* vs. C in *Sinorhizobium*), 659/746 (T–A pair vs. C–G), 747 (T vs. G), and 1012/1017 (G–C vs. A–T). Additional differences between the *Ensifer* sequences and those for all *Sinorhizobium* species except *Sinorhizobium teranga* were detected at positions 1010/1019 (A–T in *Ensifer* vs. C–G in *Sinorhizobium*) and 1011/1018 (C–G vs. G–C).

The close relatedness of *Ensifer* and *Sinorhizobium* detected by phylogenetic analyses of 16S rDNA sequences, and the high similarities between the 16S rDNA sequences of these organisms, could have implications for the status of *Ensifer* as a separate genus. *Ensifer* is not known to invade root hairs of plants and cause formation of root nodules, but this has not been tested. Similarly, *Sinorhizobium* species are not known to prey on other species of bacteria, but this has not been tested either. In addition, it is not known whether any of the unique traits of *Ensifer* are plasmid-coded and, thus, should not be included in the genus

definition. Designation of *Ensifer* as a distinct genus appears to be justified based on current information. However, it is recommended that a detailed direct comparison of *Ensifer* and *Sinorhizobium* strains be carried out to more accurately assess this situation.

ACKNOWLEDGMENTS

For the most part, this chapter is a minor revision of the original description of *Ensifer* written for the previous edition of the *Manual* by L.E. Casida, Jr. The principal changes include the addition of information from analysis of 16S rDNA sequences and the addition of data from several more recent publications. The current author thanks L.E. Casida Jr. for providing reprints and other information related to his research on *Ensifer adhaerens*. The two strains of *E. adhaerens* for which 16S rDNA sequences were determined were obtained from the American Type Culture Collection.

List of species of the genus *Ensifer*

1. ***Ensifer adhaerens*** Casida 1982, 343^{VP}
ad.haer'ens. L. adj. *adhaerens* adherent.

The characteristics are as described for the genus. Two variants have been isolated from cultures of the type strains. These variants produce less slime, which results in smaller colonies and a drier and slightly whiter appearance of the colonies.

A strain of *E. adhaerens* that differs from the type strain in its rate of attack on *M. luteus* cells but not in most other characteristics, including phage typing, was isolated from the same soil as strain A. However, the soil had been in-

cubated with added *B. subtilis* spores instead of *M. luteus* cells. This strain (designated strain 7A) has been deposited with the American Type Culture Collection under the number ATCC 33499. Strain 7A can be differentiated from strain A by its growth on desoxycholate agar; strain 7A growth remains purple throughout 10 d and then becomes whitish purple.

The habitat is soil.

The mol% G + C of the DNA is: 67 (*T_m*) and 63 (Bd).

Type strain: A, ATCC 33212.

GenBank accession number (16S rRNA): AF191739.

Genus VII. *Sinorhizobium* Chen, Yan and Li 1988b, 396^{VP} emend. de Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters and Gillis 1994, 732

L. DAVID KUYKENDALL, FAWZY M. HASHEM AND EN TAO WANG

Si.no.rhi.zo'bi.um. L. n. *sinae* China; Gr. n. *rhiza* a root; Gr. n. *bios* life; M.L. neut. n. *Sinorhizobium* which lives in a root in China; *Rhizobium* isolated in China.

Rods 0.5–1.0 × 1.2–3.0 μm. Commonly pleomorphic under adverse growth conditions. Usually contain granules of polyhydroxybutyrate, which are refractile by phase-contrast microscopy. Nonsporeforming. Gram negative. **Motile** by one polar or sub-polar flagellum or one to six peritrichous flagella. Fimbriae have been described on a few strains. **Aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor.** Optimal growth temperature, 25–30°C, but can tolerate a wide range of temperatures. Most strains can grow at 35°C; some strains can grow at 10°C, and others (*S. saheli* and *S. teranga*) can grow at temperatures up to 42–44°C (de Lajudie et al., 1994). Tolerate 1.0% NaCl, and some strains grow well on Yeast Mannitol (YM) medium containing 4.5% NaCl. Optimal pH for growth is near neutral (6–8), but some strains can grow at pH 5.0, and others tolerate pH 10.5. Colonies are circular, convex, usually opaque, sometimes translucent, raised, and mucilaginous. **Colonies are usually 2–4 mm in diameter within 3–5 days of growth on yeast mannitol-mineral salts agar** and pronounced turbidity develops after 2–3 days in aerated or agitated broth. Generation times are 3–6 h. Chemoorganotrophic, **utilizing a wide**

range of carbohydrates and salts of organic acids as carbon sources, producing acid without gas formation. Cellulose and starch are not utilized. Produce an acidic reaction in mineral-salts medium containing mannitol. Growth on carbohydrate media is often accompanied by **copious extracellular polysaccharide slime production**. Ammonium salts, nitrate, nitrite, and most amino acids can serve as nitrogen sources. **All strains require pantothenate and nicotinic acid.** Peptone is poorly utilized. Casein and agar are not hydrolyzed. **Oxidase positive.** Catalase positive. 3-Ketolactose not produced from lactose. **The organisms are characteristically able to invade the root hairs of temperate-zone and tropical-zone leguminous plants (family Leguminosae) and incite production of root nodules wherein the bacteria occur as intracellular nitrogen-fixing microsymbionts.** Strains exhibit varying degrees of host specificity, determined by the type of Nod Factors produced. In root nodules the bacteria occur as endophytes exhibiting pleomorphic forms, termed “bacteroids”, which reduce or fix gaseous atmospheric nitrogen into a combined form utilizable by the host plant.

The mol% G + C of the DNA is: 57–66.

Type species: Sinorhizobium fredii (Scholla and Elkan 1984) Chen, Yan and Li 1988b, 396 (*Rhizobium fredii* Scholla and Elkan 1984, 484.)

FURTHER DESCRIPTIVE INFORMATION

Granules of poly- β -hydroxybutyrate are common in older cells, so that upon simple staining the rods appear banded. As in the genus *Rhizobium*, all species are motile by one or more flagella. For most species examined that have multiple flagella, the insertion of the flagella is peritrichous, with random placement. Strains in those species with a single flagellum (*Sinorhizobium fredii*, *S. saheli*, *S. terangae*, and *S. xinjiangense*) are monotrichous with polar or subpolar insertion.

Cell wall composition The cell wall structure of *Sinorhizobium* is generally similar to that of other Gram-negative bacteria. The peptidoglycan consists of glutamic acid, alanine, diaminopimelic acid, and amino sugars. In addition, leucine, phenylalanine, serine, and aspartic acid have been detected in relatively large amounts in the peptidoglycan layer of several pathogenic strains. Lipopolysaccharide cell wall composition varies from strain to strain but consistently contains 2-keto-3-deoxyoctanoic acid (KDO), uronic acid, glucosamine, glucose, and galactose (Carlson, 1982).

Other genetic and physiological features of *Sinorhizobium* are generally the same as for *Rhizobium*. See the chapter on *Rhizobium*. In fact, as everyone in this area of specialization knows, much of what is known about *Rhizobium* was learned from studies on *S. meliloti*.

ENRICHMENT AND ISOLATION PROCEDURES

Like other legume-nodulating bacteria, members of the genus *Sinorhizobium* live in soils where legumes occur and may persist for years without host plants. They can be most suitably isolated from the root nodules formed on suitable host plants. Surface sterilization of root nodules is followed by sterile water rinses until the contents can be released into sterile physiological saline and streaked onto a medium with Congo red. Like *Rhizobium*, *Sinorhizobium* colonies remain white as they do not take up the dye.

MAINTENANCE PROCEDURES

Strains of *Sinorhizobium* are usually maintained on YEM medium, containing 20–50% glycerol at either -20°C or -70°C when available. Freeze-drying is also an accepted practice.

DIFFERENTIATION OF THE GENUS *SINORHIZOBIUM* FROM OTHER GENERA

See Tables BXII. α .112 and BXII. α .113 in the chapter on the genus *Rhizobium*. Apart from phenotypic characters that distinguish them as individual species, there are no common distinct characters that differentiate these species as separate from members of the genus *Rhizobium*. The closely related genus *Ensifer* has

not been tested for a variety of features unique to legume-nodulating and symbiotic nitrogen-fixing genera including these very traits and its failure to absorb Congo Red. These closely related genera can now be recognized by 16S rDNA sequencing and other molecular methods. *Sinorhizobium* can be differentiated from unrelated genera by cellular fatty acid analysis. Although *Sinorhizobium* and *Rhizobium* generally have qualitatively identical fatty acid composition, there appears to be a quantitative difference in $\text{C}_{16:0\ 3\text{OH}}$ fatty acid between *Sinorhizobium* and *Rhizobium*, in that the concentration is generally lower in *Sinorhizobium* than in *Rhizobium* (Tighe et al., 2000).

TAXONOMIC COMMENTS

The original proposal of *Sinorhizobium* Chen et al., 1988b, was questioned on the basis that there was little to discriminate this genus from *Rhizobium* (Graham et al., 1991; Jarvis et al., 1992). Subsequently, de Lajudie et al. (1994) proposed an emended description of the genus based on a comparative analysis of 16S rDNA sequences, which showed that *Rhizobium meliloti* and *Sinorhizobium fredii*, together with two new species *Sinorhizobium terangae* and *Sinorhizobium saheli*, formed a separate phylogenetic branch distinct from *Rhizobium* species (*R. leguminosarum*, *R. tropici*). Rome et al. (1996) named a sixth species, *Sinorhizobium medicae*, for strains isolated from various *Medicago* species, together with strains previously recognized as *Rhizobium meliloti* type B (Eardly et al., 1990). Two new species, *S. arboris* and *S. kostense*, were described recently from legume trees in Sudan and Kenya (Nick et al., 1999). Wang et al. (1999a) reported three new groups within this genus for isolates from *Leucaena leucocephala* in Mexico.

Inferred phylogenies based on comparative analyses of 16S rDNA sequence data show that *Sinorhizobium* species, and *Ensifer adhaerens* (Casida, 1982), a bacterial predator of bacteria in soil, form a clade(s) distinct from *Rhizobium* and *Agrobacterium* species. Apart from phenotypic characters which distinguish them as individual species, and except for the quantitative difference in $\text{C}_{16:0\ 3\text{OH}}$ fatty acid content described above, there are no common distinct characters which differentiate these species as separate from members of the genus *Rhizobium*. At the time of this writing, perhaps only about one-half of the investigators in the field accept the taxonomic validity of *Sinorhizobium*. This new genus, together with *Ensifer*, will probably not stand if supporting data that provide evidence of distinct taxa cannot be gathered. The present fragile nature of these genera will be further undermined when strains from different soils and hitherto untested legume/*Rhizobium* combinations are found to be intermediate between the genera.

The taxonomic relationships of *Sinorhizobium* are discussed fully and further descriptive information is given in the chapter on *Rhizobium*. Perhaps the ultimate tool of taxonomy, the complete genome sequence for *S. meliloti*, was recently reported by Galibert et al. (2001).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *SINORHIZOBIUM*

See Tables BXII. α .112 and BXII. α .113 in the chapter on the genus *Rhizobium*. Reports of specific phenotypic differences are needed for some species. The species of *Sinorhizobium* can also be differentiated by comparative sequence analysis of 16S rRNA

gene sequence data. Fatty acid analysis can also be useful to discriminate *Sinorhizobium* from other related genera (Jarvis et al., 1996).

List of species of the genus Sinorhizobium

1. ***Sinorhizobium fredii*** (Scholla and Elkan 1984) Chen, Yan and Li 1988b, 396^{VP} (*Rhizobium fredii* Scholla and Elkan 1984, 484.)

fredii M.L. gen. n. *fredii* of E.B. Fred.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Motile by one polar flagellum or by 1–3 peritrichous flagella. Relatively fast growing: colonies are 2–4 mm in diameter after 3–5 days growth on yeast extract mannitol agar. Temperature growth optimum is 25–30°C. Most strains grow at 35°C, and some grow at 10°C. Optimal pH for growth, 6–8; range pH 5.0–10.5. Most strains grow on YMA containing 1.0% NaCl, but a few strains tolerate >3.0% NaCl. Require pantothenate and nicotinate as growth factors. Utilize relatively few organic substrates as sole sources of carbon. Two chemovars were proposed—*fredii* and *siensis*—that were differentiated by the ability of *siensis* to reduce litmus and produce a final pH in growth media greater than 6.1, and its resistance to kanamycin (20 ppm). Later revisions have led to a consolidation of these groups. *S. fredii* can be differentiated from the other *Sinorhizobium* species by auxanographic characteristics, protein SDS-PAGE profile, DNA–DNA reassociation, and comparative analysis of 16S rDNA sequence data.

This species effectively nodulates *Cajanus cajan*, *Glycine max* cv. Peking, *Glycine soja*, and *Vigna unguiculata*. Dowdle and Bohlool (1985) reported new strains that are symbiotically competent with North American cultivars of soybean. Some original strains, including the type strain, were also found to nodulate *Medicago sativa* (Hashem et al., 1997) with efficient symbiotic nitrogen fixation (Kuykendall et al., 1999).

The mol% G + C of the DNA is: 60–64 (T_m).

Type strain: PRC 205, ATCC 35423, ICMP 11139, NCIB 12104, USDA 205.

GenBank accession number (16S rRNA): D01272, X67231.

2. ***Sinorhizobium arboris*** Nick, de Lajudie, Eardly, Suomalainen, Paulin, Zhang, Gillis and Lindström 1999, 1366^{VP} *arbo. ris*. L. fem. n. *arbor* tree; n. *arboris* of the tree.

Short, aerobic, Gram-negative rods that are motile by one or two polar or subpolar flagella. Maximum growth temperature is about 42°C. On YEM medium, most strains produce circular, cream-colored, semi-translucent colonies that become very mucilaginous and often spread over the entire plate within 2–4 d. Utilize a wide range of carbon sources and amino acids for growth. Grow on L-isoleucine and tolerate 3% (w/v) NaCl, heavy metals (but not Al), and antibiotics. Produce melanin and grow at pH 8.5. However this species cannot be identified by physiological features and biochemical traits alone. Nodulates *Acacia senegal* and *Prosopis chilensis*. SDS-PAGE whole protein pattern, MLEE, rep-PCR genomic fingerprinting, RFLP analysis of amplified 16S rRNA, total DNA–DNA hybridization, and 16S rDNA sequence comparison are useful for distinguishing closely related species at the molecular level.

The mol% G + C of the DNA is: 60.6–61.8 (T_m).

Type strain: HAMBI 1552, LMG 14919.

GenBank accession number (16S rRNA): Z78204.

3. ***Sinorhizobium kostiense*** Nick, de Lajudie, Eardly, Suomalainen, Paulin, Zhang, Gillis and Lindström 1999, 1366^{VP} *kos. ti. en' se*. L. neut. adj. *kostiense* pertaining to Kosti, the region in Sudan where most of these organisms have been isolated.

Short, aerobic, Gram-negative rods that are motile by one or two polar or subpolar flagella. Maximum growth temperature is about 38–40°C for most strains. On YEM medium, most strains produce circular, cream-colored, semi-translucent colonies that become very mucilaginous and often spread over the entire plate within 2–4 d. Utilize a relatively narrow range of carbon sources and amino acids for growth. Tolerate 1% (w/v) NaCl; sensitive to heavy metals except lead and copper. Sensitive to most antibiotics. Produce melanin but fail to grow at pH 5.5 or 8.5. This species cannot be identified by physiological features and biochemical traits alone. Nodulates *Acacia senegal* and *Prosopis chilensis*. SDS-PAGE whole protein pattern, MLEE, rep-PCR genomic fingerprinting, RFLP analysis of amplified 16S rRNA, total DNA–DNA hybridization, and 16S rDNA sequence comparison are useful for distinguishing closely related species at the molecular level.

The mol% G + C of the DNA is: 57.9–61.6 (T_m).

Type strain: HAMBI 1489, LMG 15613.

GenBank accession number (16S rRNA): Z78203.

4. ***Sinorhizobium medicae*** Rome, Fernandez, Brunel, Normand and Cleyet-Marel 1996, 979^{VP} *me' di. cae*. L. fem. n. *medica* from *medica lucerne* (plant belonging to the genus *Medicago*); L. gen. n. *medicae* of *medica*.

The characteristics are as described for the genus and as listed in Table BXII.α.112 in the chapter on the genus *Rhizobium*. Fast growing: semi-translucent, circular, and mucoid colonies spread over the plate in 3–5 days on yeast extract mannitol agar. Temperature growth optimum, 25–30°C. Most strains grow at 35°C but not at 42°C; 40°C is the maximum temperature for growth. Optimal pH for growth, 6–8; range pH 5.0–10. Grows in media containing 2% NaCl but does not grow well in media containing 3% NaCl. Resistant to nalidixic acid. Differentiated from other *Sinorhizobium* and *Rhizobium* spp. based on differences in 16S rDNA sequence data. Sequence similarities indicate a very close relationship to *S. meliloti* (99.7%).

Strains have been isolated from various *Medicago* species in different geographical sites including southern France and the eastern Mediterranean basin, which are in the center of origin of the genus *Medicago*. Isolated from *Medicago orbicularis*, *M. polymorpha*, *M. rugosa*, and *M. truncatula*. *Sinorhizobium medicae* strains fix nitrogen with *M. polymorpha*, whereas *S. meliloti* forms ineffective nodules.

The mol% G + C of the DNA is: 61.0–63.0 (T_m).

Type strain: A 321, USDA 1037.

GenBank accession number (16S rRNA): L39882.

5. ***Sinorhizobium meliloti*** (Dangeard 1926) de Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters, and Gillis 1994, 731^{VP} (*Rhizobium meliloti* Dangeard 1926, 194.)

me. li. lo' ti. M.L. masc. n. *Melilotus* generic name of sweet clover; M.L. gen. n. *meliloti* of *Melilotus*.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Motile by 2–6 peritrichous flagella. Variable growth on YMA containing 2% NaCl. Variable growth at 39–40°C. Does not require thiamine or pantothenate for growth; some strains require biotin. pH range for growth, 5.0–9.5. Utilizes a relatively wide range of organic substrates as sole sources of carbon. Distinguished from other *Sinorhizobium* and *Rhizobium* species by DNA–DNA reassociation data, differences in protein profiles obtained by SDS-PAGE, and 16S rDNA sequence comparison. Sequence similarities indicate a close relationship to *S. medicae*. Two subpopulations have been distinguished by DNA hybridization and multilocus enzyme electrophoresis.

Forms nitrogen-fixing nodules on *Melilotus*, *Medicago*, and *Trigonella*. Gao and Yang (1995) reported a Chinese *Sinorhizobium meliloti* strain that nodulated and fixed nitrogen in association with both alfalfa and soybean. Well characterized genetically. Symbiosis-controlling genes are carried on megaplasmids. In addition, strains harbor various numbers (0–4) of large pRme plasmids (90–500 kb) (Boivin et al., 1997).

The mol% G + C of the DNA is: 62–63 (T_m).

Type strain: ATCC 9930, ICMP 12623, LMG 6133, USDA 1002.

GenBank accession number (16S rRNA): X67222.

6. ***Sinorhizobium saheli*** de Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters, and Gillis 1994, 732^{VP}

sa' hel.i. N.L. gen. n. *saheli* of the Sahel, the region in Africa from which they were isolated.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Motile by one or more polar or subpolar flagella. Colonies can be mucoid and spreading on yeast extract mannitol agar. Old colonies become brown. Can grow at 44°C. Utilizes a range of organic substrates as sole sources of carbon (Table BXII.α.113 in the chapter on the genus *Rhizobium*). Distinguished from other *Sinorhizobium* and *Rhizobium* species by DNA–DNA reassociation data, differences in protein profiles obtained by SDS-PAGE, and 16S rDNA sequence comparison. Haukka et al. (1998) reported two different sequences for a 230-nucleotide segment of the 16S ribosomal RNA gene in the type strain of *S. saheli*.

Nodulates different *Sesbania* species (*S. cannabina*, *S. grandiflora*, *S. rostrata*, *S. pachycarpa*) growing in the Sahel; in addition to these species, strains can nodulate *Acacia seyal*, *Leucaena leucocephala*, and *Neptunia natans*. Can form effective stem nodules on *Sesbania rostrata* when plants are not previously nodulated on their roots (Boivin et al., 1997).

The mol% G + C of the DNA is: 65–66 (T_m).

Type strain: ORS 609, DSM 11273, ICMP 13648, LMG7837.

GenBank accession number (16S rRNA): X68390.

7. ***Sinorhizobium terangae*** de Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters, and Gillis 1994, 732^{VP} (*Sinorhizobium teranga* (sic) de Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters, and Gillis 1994, 732.)

te' ran.gae. N.L. gen. n. *terangae* of *teranga* hospitality, in the language of West African Wolof people; referring to the isolation of this species from different host plants.

The characteristics are as described for the genus and as listed in Tables BXII.α.112 and BXII.α.113 in the chapter on the genus *Rhizobium*. Motile by one or several polar or subpolar flagella. Colonies are mucoid and spreading on yeast extract mannitol agar. Old colonies become brown. Can grow at 44°C. Utilizes a range of organic substrates as sole sources of carbon (Table BXII.α.113 in the chapter on the genus *Rhizobium*). Differentiated from other *Sinorhizobium* and *Rhizobium* species by DNA–DNA reassociation data, differences in protein profiles obtained by SDS-PAGE, and 16S rDNA sequence comparison.

Nodulates *Acacia* species (*A. senegal*, *A. laeta*, *A. tortilis* subsp. *raddiana*, *A. horrida*, *A. mollissima*) and *Sesbania* species (*S. rostrata*, *S. cannabina*, *S. aculeata*, *S. sesban*). Two biovars were distinguished based on the host range of strains (Lortet et al., 1996): *S. terangae* biovar *sesbaniae* for strains able to nodulate *Sesbania* species (*S. grandiflora*, *S. pubescens*, and *S. rostrata*) but not *Acacia* species (*A. senegal*, *A. tortilis* subsp. *raddiana*, and *A. nilotica*) nor *Leucaena leucocephala*; *S. terangae* biovar *acaciae* for strains able to nodulate *Acacia* species (*A. senegal*, *A. tortilis* subsp. *raddiana*, and *A. nilotica*) and *Leucaena leucocephala* but not *Sesbania* species (*S. grandiflora*, *S. pubescens*, and *S. rostrata*).

The mol% G + C of the DNA is: 60.8–61.6 (T_m).

Type strain: ORS 1009, DSM 11282, ICMP 13649, LMG 7834.

GenBank accession number (16S rRNA): X68391.

8. ***Sinorhizobium xinjiangense*** Chen, Yan and Li 1988b, 396^{VP} (*Sinorhizobium xinjiangensis* Chen, Yan and Li 1988b, 396.) *xin.jian.gen'se.* M.L. adj. *xinjiangense* pertaining to the suburbs of Xinjiang, China.

The characteristics are as described for the genus and as listed in Table BXII.α.112 in the chapter on the genus *Rhizobium*. Motile by one polar flagellum or by 1–3 peritrichous flagella. Relatively fast growing: colonies are 2–4 mm in diameter after 3–5 days growth on yeast extract mannitol agar. Temperature growth optimum, 25–30°C. Most strains grow at 35°C and some grow at 10°C. Optimal pH for growth, 6.0–8.0; range, 5.0–10.5. Does not grow on YMA containing 1.5% NaCl, but some strains tolerate 4.5% NaCl.

The species was proposed based on nutritional and biochemical differences from *R. leguminosarum*, *S. fredii*, *S. meliloti*, *Agrobacterium tumefaciens*, and *A. rhizogenes*. 16S rDNA sequence similarities indicate a close relationship to *S. fredii*. The species differs from *S. fredii* in that, unlike *S. fredii*, *S. xinjiangense* cannot grow at low and high pH (<5.5 and >8.5 respectively). All strains of *S. xinjiangense* produce acid in litmus milk, and are sensitive to a number of antibiotics such as vancomycin (25 µg/ml), chloramphenicol (125 µg/ml), penicillin (25 µg/ml), and streptomycin (5 µg/ml). Isolated from *Glycine max*.

The mol% G + C of the DNA is: 60–64 (T_m).

Type strain: ATCC 49357, CCBau 110, IAM 14142, ICMP 11141.

GenBank accession number (16S rRNA): D12796.

Family II. **Bartonellaceae** Gieszczykiewicz 1939, 25^{AL}

DAVID F. WELCH

Bar.to.nel.la'ce.ae. M.L. fem. n. *Bartonella* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Bartonellaceae* the *Bartonella* family.

Small coccobacilli, may be beaded or filamentous and $<3\ \mu\text{m}$ in their greatest diameter. Erythrocytic forms stain lightly with many aniline dyes but distinctly with Giemsa's stain after methanol fixation. Gram negative, not acid-fast. Some species have polar flagella. Cultivable, but highly fastidious, *in vitro*, on blood-enriched bacteriological media. Arthropod transmission has been established. Etiological agents demonstrable in diverse clinical material from humans and erythrocyte-associated bacteria of other vertebrates. Cause bartonellosis, cat scratch disease, bacillary angiomatosis, endocarditis, trench fever, and a spectrum of other human inflammatory lesions.

On the basis of 16S rRNA gene sequence analysis, the family *Bartonellaceae* is in the *Alphaproteobacteria*. The genus *Bartonella* was removed from the order *Rickettsiales* and the family *Rickettsiaceae*, resulting in a single genus within the family *Bartonellaceae* (Brenner et al., 1993). The genus *Rochalimaea*, then contained within the family *Rickettsiaceae*, was at the same time combined with *Bartonella*, and subsequently *Grahamella* was merged with *Bartonella* (Birtles et al., 1995).

Type genus: **Bartonella** Strong, Tyzzer and Sellards 1915, 808 emend. Birtles, Harrison, Saunders and Molyneux 1995, 7.

Genus I. **Bartonella** Strong, Tyzzer and Sellards 1915, 808^{VP} emend. Birtles, Harrison, Saunders and Molyneux 1995, 7

DAVID F. WELCH

Bar.to.nel'la. M.L. dim. ending *-ella*; M.L. fem. dim. n. *Bartonella* named after Alberto L. Barton, who described these organisms in 1909, after studying the agent of Carrion's disease.

Morphology is small ($0.5\text{--}0.6 \times 1.0\ \mu\text{m}$), **slightly curved bacillus**. Not acid-fast. **Faintly stains Gram negative** (stains poorly or not at all with many aniline dyes) but satisfactorily with Romanowsky's or Giemsa's stain. Warthin-Starry silver staining of fixed tissue sections reveals bacilli in clusters. May be seen in stained blood films appearing as rounded or ellipsoidal forms or as slender, straight, curved, or bent rods, occurring singly or in groups. They characteristically occur in chains of several segmenting organisms, sometimes swollen at one or both ends and frequently beaded. In the tissues, they are situated **within the cytoplasm of endothelial cells** as isolated elements or are grouped in rounded masses. **Intraerythrocytic forms occur** in the blood of felines, small rodents, birds, fish, and other animals. In cultures, the cells may be very **autoadherent**. Some species possess unipolar flagella. **The presence of pili** is associated with the marked adherence and may mediate specific interaction with host endothelial cells and erythrocytes leading to intracellular localization.

Aerobic but **highly fastidious**. May be cultivated on media enriched with blood components in the presence of air or 5% CO₂. **Growth occurs at 20–37°C after prolonged incubation** (7–21 d). No growth on MacConkey or nutrient agars. Cocultivation with an endothelial cell line can also be performed and this method may be more successful in recovering organisms from specimens such as tissue. There are **numerous reservoirs and vectors** for *Bartonella* spp. The organisms are transmitted by arthropod vectors (*Lutzomyia verrucarum*, *Pediculus humanus*, *Ctenocephalides felis* [*Siphonaptera pulicidae*], and possibly ticks); *B. bacilliformis* is found only in the Andes region of South America. Etiological agents of human bartonellosis, cat scratch disease, bacillary angiomatosis, peliosis hepatis, trench fever, endocarditis, and neuroretinitis. **Six species cause Bartonella-associated infectious diseases in humans**. The organisms are usually catalase and oxidase negative, aerobic, and they do not produce acid from carbohydrates. *Bartonella* spp. have relatively simple gas-liquid chromatography profiles consisting mainly of C_{18:1}, C_{18:0}, and C_{16:0} acids. A portion of the 16S–23S rRNA intergenic spacer region can be targeted with primers that can distinguish *Barto-*

nella from other genera including those closely related within the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 37–41.

Type species: **Bartonella bacilliformis** (Strong, Tyzzer, Brues, Sellards and Gastiaboru 1913) Strong, Tyzzer and Sellards 1915, 808 (*Bartonella bacilliformis* Strong, Tyzzer, Brues, Sellards and Gastiaboru 1913, 1715.)

FURTHER DESCRIPTIVE INFORMATION

Stained blood films have been used to detect intraerythrocytic *B. bacilliformis* in patients with Oroya fever. Bacilli may be demonstrated by use of the Warthin-Starry silver stain during the early stages of lymphadenopathy in cat scratch disease due to *B. henselae*, but typically not during the later granulomatous stage of inflammation. When seen in blood smears, *B. bacilliformis* consists of small, polymorphic forms. The maximum morphological range is seen in the blood of man, where the organisms appear as red-violet rod or coccil forms situated on or in the red cells when stained with Giemsa's stain. Bacilliform bodies are the most typical, measuring $0.25\text{--}0.5 \times 1.0\text{--}3.0\ \mu\text{m}$. The cells are often curved and may show polar enlargement and granules at one or both ends. Rounded organisms measure $\sim 0.75\ \mu\text{m}$ in diameter and a ring-like variety is sometimes abundant. By light microscopy or by "stripping" in the pseudoreplica technique for electron microscopy (Peters and Wigand, 1955), the organisms appear to be situated on the surface of erythrocytes; however, they have also been reported to occur within erythrocytes in thin sections observed by electron microscopy (Cuadra and Takano, 1969). The Gram stain of a colony from solid media reveals small, Gram-negative, slightly curved rods resembling *Campylobacter*, *Helicobacter*, or *Haemophilus*. Cells, especially of *B. henselae*, are very autoadherent, as can be demonstrated by attempting to scrape colonies off a culture plate with a loop. In semisolid media, a mixture of rods and granules appears. The organisms may occur singly or in large and small, irregular, dense collections measuring up to $25\ \mu\text{m}$ or more in length. Punctiform, spindle-

shaped, and ellipsoidal forms that vary in size from $0.2\text{--}0.5 \times 0.3\text{--}3.0 \mu\text{m}$ also occur.

The organisms have a trilaminar cell wall, the formation of which can be inhibited by penicillin. The cellular fatty acid composition among *Bartonella* spp. is relatively simple, compared to that of many other Gram-negative bacteria, in that they have gas-liquid chromatography profiles consisting mainly of $C_{18:1}$, $C_{18:0}$, and $C_{16:0}$ acids. *B. elizabethae* and *B. vinsonii* contain a greater amount of $C_{17:0}$ than the other species. *B. bacilliformis* contains a significant amount of $C_{16:1}$. An unusual branched-chain fatty acid (11-methyloctadec-12-enoic acid) is found in *Afipia* spp. but not in *Bartonella* spp., and *Brucella* spp. contain a relatively large amount of $C_{19:0}$ cyclopropane acids. In culture, the cells of two species (*B. bacilliformis* and *B. clarridgeiae*) possess a tuft of 1–10 unipolar flagella (Peters and Wigand, 1955; Lawson and Collins, 1996a). Other species, especially *B. henselae*, *B. alsatica*, and *B. tribocorum*, have pili. Phenotypically similar to type 4 pili, they mediate adherence to and entry into human epithelial cells (Batterman et al., 1995). Flagella have not been demonstrated in tissues, but the pili have been.

Bartonella spp. demonstrate various colonial morphologic types, from clear (*B. bacilliformis*) to smooth white (*B. quintana* and *B. alsatica*) to rough tan (*B. henselae* and *B. tribocorum*). Several species embed in the agar. Colonies of *B. henselae* are typically of two types: (i) irregular, raised, whitish, rough (cauliflower or molar tooth or verrucous), and dry in appearance or (ii) smaller, circular, tan, and moist in appearance, tending to pit and adhere to the agar. Both types are usually present in the same culture (Fig. BXII.α.137). The degree of colonial heterogeneity varies by species and by strain, and is probably related to the degree of piliation. Repeated subcultures of *B. henselae* tend to have increasing proportions of smooth colonies. *B. quintana* may appear as uniformly smooth colonies in primary cultures. Cultures of *B. henselae* on blood agar may produce an odor similar to the caramel odor (diacetyl) produced by *Streptococcus milleri*.

The majority of isolates require more than 7 days of incubation before they can be detected by culture. A source of blood

or hemoglobin in the medium is necessary in most cases. *B. bacilliformis* may be cultivated in semisolid agar containing fresh rabbit serum and rabbit hemoglobin or containing the blood of human, horse, or rabbit, with and without the addition of fresh tissue and carbohydrates. It may also be cultivated in other culture media containing blood, serum, or plasma, in Huntton's hormone agar, in semisolid gelatin media, and in blood-glucose-cysteine agar. It can also be grown in certain tissue cultures and in the chorioallantoic fluid and yolk sac of the chicken embryo. The other species can be recovered on blood or chocolate agar media as well.

Bartonella spp. are typically inert biochemically. In conventional carbohydrate utilization tests no acid or gas production occurs from amygdalin, L-arabinose, dextrin, dulcitol, fructose, D-galactose, D-glucose, inulin, lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, salicin, sucrose, trehalose, or D-xylose. Gelatin is not liquefied. Esculin is not hydrolyzed. H_2S is not detected with lead acetate.

Bartonella is a monophyletic genus within the *Alphaproteobacteria*. Species of *Bartonella* are animal cell associated but closely related to the plant cell associated genera, *Agrobacterium* and *Rhizobium*. Common pathogenic domains necessary for invasion and survival in association with cells have been preserved in the chromosomes of both the animal- and plant-associated *Alphaproteobacteria*. These have evolved through reductions of the larger genomes, including a second chromosome in some instances, of chemoautotrophic ancestors (Moreno, 1998). There is also a high level (~95%) of similarity between *Bartonella* and *Brucella* according to 16S rRNA gene sequence analysis (Brenner et al., 1993). Phylogenetic analysis of *Bartonella* species can be done based on either 16S rRNA or citrate synthase (*gltA*) gene sequencing. The latter has been compared to 16S rRNA gene sequence analysis and some investigators (Birtles and Raoult, 1996) believe the *gltA*-derived phylogeny is more useful than the phylogeny derived from 16S rDNA sequence data for investigating the evolutionary relationships of *Bartonella* species. The *gltA* method may result in amplification of two products of differing

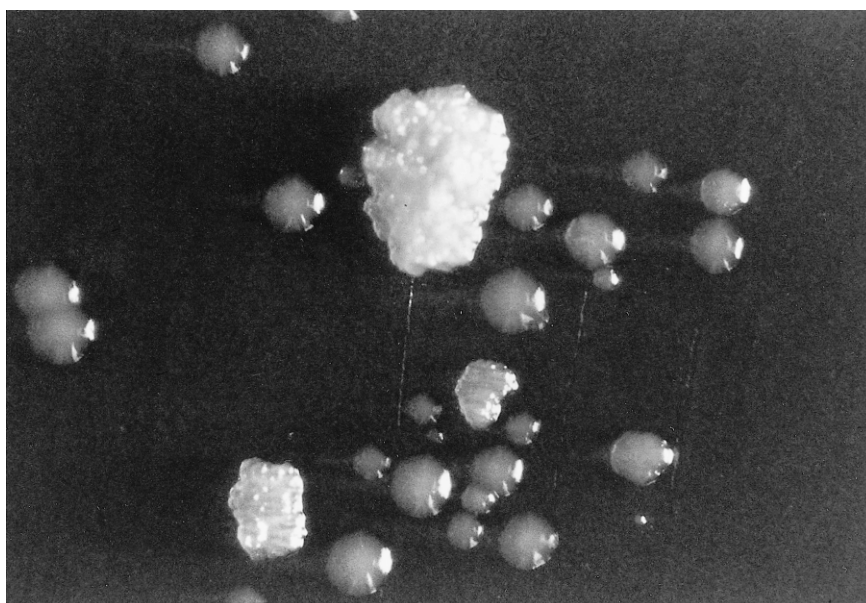


FIGURE BXII.α.137. Colonies of *Bartonella henselae* (40× magnification), showing heterogeneous mixture of smooth and rough types.

sizes from *B. bacilliformis* (Birtles, 1995). Limited work with heat shock protein (groEL) genes of some *Bartonella* species has also been conducted, leading to similar phylogenetic conclusions (Haake et al., 1997).

A portion of the 16S rDNA consisting of 241 nucleotides can be amplified using primers p24E and p12B (Relman et al., 1990a). Signature sequences, e.g., a 3 base pair difference between *B. quintana* and *B. henselae* (Koehler et al., 1992), occur within this fragment.

A genus-specific primer set targeting the internal portions of the 16S–23S intergenic spacer region can distinguish *Bartonella* species from other *Alphaproteobacteria*, including *Brucella abortus*, *Agrobacterium tumefaciens*, and *Rhizobium meliloti* (Minnick and Barbican 1997). These investigators also developed species-specific primers. Distinguishing among isolates of the same species using DNA fingerprinting methods has been applied mainly with *B. henselae*. Pulsed-field gel electrophoresis (PFGE), 16S rRNA type-specific PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP) PCR, and arbitrarily primed (AP)-PCR have all been used to clearly distinguish several subtypes (Sander et al., 1998). These methods provide useful epidemiological tools and demonstrate a high degree of genetic heterogeneity.

FtsZ proteins (the highly conserved cell division protein) of *B. bacilliformis*, *B. henselae*, and *B. quintana* are about twice as large as the FtsZ proteins reported in most other organisms. The amino acid sequences of *B. henselae* and *B. quintana* ftsZ are 81–83% identical to the corresponding protein in *B. bacilliformis*, suggesting that localized differences within the sequence of the *Bartonella* ftsZ genes may be used as the basis for species-specific identification (Kelly et al., 1998b). *B. henselae* harbors a bacteriophage that has been cloned and sequenced (Anderson et al., 1997; Bowers et al., 1998). Associated with the bacteriophage is a 31-kDa major protein (Pap31) that is targeted to the cell membrane. *B. vinsonii* subsp. *berkhoffii* contains a 12-base insertion sequence in the 16S rRNA gene that is not present in other *Bartonella* species (Kordick et al., 1996).

The FtsZ protein is antigenic in *B. bacilliformis* and in *B. henselae* due to its partial exposure at the cell surface (Padmalayam et al., 1997). Antigenicity is species and strain specific as indicated by the lack of cross-protection between *B. henselae* and *B. clarridgeiae* and the lack of protection between *B. henselae* types I, II, and a wildlife strain (Yamamoto et al., 1998). On the other hand, serological cross-reactions do occur between species and with the genera *Chlamydia* and *Bartonella*. Maurin et al. (1997) reported eight patients originally diagnosed as having *Chlamydia pneumoniae* endocarditis whose sera reacted with *B. quintana* antigens in a microimmunofluorescence technique. Adsorption of sera with *B. quintana* or *C. pneumoniae* antigens removed anti-*C. pneumoniae* antibodies, whereas adsorption with *C. pneumoniae* antigens did not change antibody titers to *B. quintana*. There is also cross reactivity between *B. henselae* and *Coxiella*. La Scola and Raoult (1996b) demonstrated that >50% of chronic Q fever patients they studied had antibodies that reacted significantly against *B. henselae* antigen.

Agglutination of suspensions of *B. bacilliformis* by sera from convalescent patients has been reported. Immune sera fix complement in the presence of the organisms. When various isolates have been employed, no significant titer differences have been found in quantitative tests. Immune rabbit sera do not agglutinate *Proteus* strains OX19, OX2, or OXK at titers above 1:20. Serodiagnosis of cat scratch disease by indirect immunofluores-

cence or enzyme immunoassay is a practical technique and carries a sensitivity of approximately 85% and a specificity of approximately 95%. IgM reactivity is directed to an 8-kDa band and IgG to 209-, 208.5-, 208-, 116-, and 80-kDa bands by Western blot analysis of *B. henselae* serum samples (Litwin et al., 1997).

Bartonella spp. generally display high levels of *in vitro* susceptibility to antibacterial agents. Agar dilution testing shows resistance only to fosfomycin, colistin, and vancomycin (Maurin et al., 1995). *B. bacilliformis* is resistant *in vivo* to neosalvarsan and to other arsenical compounds in general. It is sensitive to penicillin, streptomycin, chloramphenicol, and oxytetracycline. Oral chloramphenicol is the standard treatment for *B. bacilliformis* infection. When grown with penicillin, the organism produces L forms (Sharp, 1968). *B. henselae*, *B. quintana*, and *B. elizabethae* are susceptible to the macrolides azithromycin, clarithromycin, dirithromycin, erythromycin, and roxithromycin based on testing in Vero cell cultures (Ives et al., 1997). Agents that should be considered in therapy of cat scratch disease include azithromycin or rifampin. In the initial antimicrobial treatment of hepatosplenic cat-scratch disease in children, rifampin therapy, initiated alone or in combination, results in improvement within 1–5 days (Arisoy et al., 1999). A placebo-controlled clinical study showed that azithromycin caused an 80% decrease in initial lymph node volume in 7 of 14 azithromycin-treated cat scratch disease patients during the first 30 days of observation (Bass et al., 1998). Erythromycin, doxycycline, or ciprofloxacin is recommended for therapy of bacillary angiomatosis.

B. bacilliformis, *B. quintana*, *B. henselae*, and *B. elizabethae* are the primary pathogenic species in humans. *B. clarridgeiae*, originally isolated from a domestic cat, may cause lymphadenopathy in humans (Kordick et al., 1997). *B. vinsonii* is potentially pathogenic, as documented by a human isolate of *B. vinsonii* subsp. *arupensis* (Welch et al., 1999). *B. henselae* and *B. clarridgeiae* were discovered during the 1990s, largely in the course of testing for opportunistic infections in human immunodeficiency virus (HIV)-infected individuals. In recent years, *B. quintana* bacteremic infection (trench fever) in the absence of HIV has been identified sporadically, mainly in homeless persons in North America and Europe, but it was a major cause of morbidity during WW I. There is archeological evidence for pre-Colombian existence of bartonellosis due to *B. bacilliformis* in South America. The spectrum of diseases caused by *Bartonella* spp. other than *B. bacilliformis* includes trench fever, cat scratch disease, bacillary angiomatosis, bacillary peliosis, relapsing bacteremia, endocarditis, lebers neuroretinitis, and aseptic meningitis (Wong et al., 1995a; Raoult et al., 1996). The mechanism of pathogenesis by which *Bartonella* spp. cause disease is not fully understood. A central feature is an effect that causes proliferation of microvascular endothelial cells and neovascularization (angiogenesis). Surface-exposed determinants such as adhesins and other outer membrane proteins are of substantial importance in the pathogenesis (Burgess and Anderson, 1998). A process of adherence, followed by invasion of feline erythrocytes, is involved in establishing long-term bacteremia in cats (Mehock et al., 1998). Intracellular localization of *B. quintana* has been demonstrated *in vivo* and within endothelial cell cultures (Brouqui and Raoult, 1996). Upon engulfment by human endothelial cells, the organisms appear in vacuoles similar to the morulae seen in ehrlichiae- or chlamydiae-infected cells. *B. vinsonii* subsp. *vinsonii*, *B. grahamii*, *B. taylorii*, and *B. doshiae* were isolated from small wild mammals. *B. vinsonii* subsp. *berkhoffii* was isolated from a dog with endocarditis and *B. vinsonii* subsp. *arupensis* was isolated from

naturally infected mice and a man with a febrile illness. Human bartonellosis is a classically biphasic disease caused by *B. bacilliformis* and manifested as a progressive anemia (Oroya fever) followed by a cutaneous eruption (Verruga Peruana). While typical bartonellosis has remained endemic for the past century in highland provinces near the Peruvian border, there have recently been an increasing number of atypical cases in which monophasic verrucous cutaneous disease is the only clinical manifestation (Amano et al., 1997). These cases consist of mild clinical disease, possibly associated with less virulent bacterial strains.

The transmission of Oroya fever is dependent on the ecology of the sand fly (*Phlebotominae*: *Lutzomyia verrucarum*) vector and is therefore typically confined to an elevation of 2500–8000 feet above sea level in a band less than 100 miles wide and ~1000 miles long on the western slopes of the Andes mountains in Peru, Ecuador, and Colombia. However, the atypical cases tend to now be disseminating or re-emerging in previously disease-free areas.

Experimental Oroya fever has not been successfully produced in animals, except rarely in an atypical form in monkeys. Experimental Verruga Peruana has been produced in man and in a number of species of monkeys. The other species of *Bartonella* are less geographically confined. The vector of *B. quintana* is *Pediculus humanus*, the human body louse. Outbreaks of trench fever (also known as Volhynia fever, Meuse fever, His-Werner disease, shinbone fever, shank fever, and quintan or five-day fever) therefore occur focally, often associated with conditions of poor sanitation and personal hygiene that may predispose to exposure to lice. Nonhuman vertebrate reservoirs have not been identified for *B. bacilliformis*, *B. quintana*, or *B. elizabethae*. No arthropod vector has been identified for *B. elizabethae*. *B. henselae* or *B. clarridgeiae* can induce chronic infection in specific-pathogen-free cats (Guptill et al., 1998; Kordick et al., 1999). There may be minimal clinical signs but histological changes occur and *Bartonella* DNA can be detected in tissues, supporting an etiologic role for *Bartonella* species in idiopathic diseases of cats. *B. henselae* also causes reproductive failure in female cats, but has not been found to be vertically transmitted. In contrast, evidence does suggest the possibility of vertical transmission of *Bartonella* spp. among natural rodent hosts (Kosoy et al., 1998). Stray cats are a major reservoir of *Bartonella* spp., which can be transmitted to pet cats and, consequently, to humans (Heller et al., 1997). It has been demonstrated that the cat flea readily transmits *B. henselae* to cats (Chomel et al., 1996). Cats are also the reservoir for *B. clarridgeiae* and *B. koehlerae*. Mice, voles, and rats carry subspecies of *B. vinsonii*. *Bartonella* can be detected in various rodent populations, most of which are also reservoirs of tick-borne pathogens such as *Borrelia burgdorferi*, *Ehrlichia* spp., and *Babesia microti* (Kosoy et al., 1997; Hofmeister et al., 1998). These observations, along with the case reports of bacteremia due to *B. henselae* in men who had sustained tick bites prior to their illnesses (Lucey et al., 1992), suggest that *Bartonella* spp. may also be transmitted by ticks. Schouls et al. (1999) have also found *Bartonella* DNA in a high proportion of an *Ixodid* tick population.

ENRICHMENT AND ISOLATION PROCEDURES

The specimen source of most isolates of *Bartonella* is blood or tissue. The fastidious nature of these organisms requires that precautions be taken to minimize delayed attempts at isolation. If storage of specimens is necessary, they should be kept frozen. Blood collected in tubes containing EDTA can be plated after 26 d at –65°C with no loss of sensitivity (Brenner et al., 1997). Blood-lysis tubes (Isolator; Wampole, Cranbury, NJ) yield good

recovery from freshly collected specimens (Welch et al., 1992). *Bartonella* spp. have been isolated using agar, in semisolid media, and in broth. Broth-based systems generally tend to have lower sensitivity. If a broth-based blood culture system that relies on CO₂ detection to indicate growth is used, it should be combined with acridine orange staining at the end of a 7-d incubation and subculture to solid media (Spach et al., 1995). Combining the subculture of blood culture broth into shell vials has been reported to produce sensitivity of 71% for recovery of *B. quintana* or *B. henselae* from patients with endocarditis, bacillary angiomatosis, and lymph nodes of cat scratch disease and no prior antibiotic therapy (La Scola and Raoult, 1999b). The sensitivity of culture was still low when compared with that of PCR-based detection or serological methods of diagnosis. A modified RPMI 1640 medium has also been described by Wong et al. (1995b) for recovery of *B. henselae* from both tissue and blood. Isolator-processed blood should be plated on enriched (chocolate- or blood-containing) medium incubated at 35–37°C (30°C for *B. bacilliformis*) under conditions of 5–10% CO₂ and >40% humidity. For optimal recovery, the medium should be as freshly prepared as possible. Plates should be sealed after the first 24 h of incubation to preserve the moisture content of the medium. Isolates have been obtained from liver, spleen, lymph node, and skin after homogenization either by direct plating or by cocultivation with an endothelial cell line (Koehler et al., 1992). The cocultivation method may be more successful in recovering organisms from tissue specimens. Selective culture techniques have not been developed, so recovery of isolates from contaminated specimens may not be possible. During Oroya fever, the organisms can be isolated from blood and from endothelial cells of lymph nodes, spleen, and liver. In cases of Verruga Peruana, they are found in the blood and in the eruptive lesions. The organisms can also be isolated from the sand fly vector (*Phlebotomus* spp.). Other species have also been isolated from their respective arthropod vectors, and *B. henselae* is readily isolated from the blood of cats. In human cases of cat scratch disease, attempts at isolating the organism are rarely successful.

MAINTENANCE PROCEDURES

Viability of cultures can usually be maintained by passage on blood or chocolate agar at biweekly intervals. During serial transfers, the greatest longevity of *B. bacilliformis* is achieved at a temperature of 28°C. Cultures of this and the other species are best maintained during storage at –70°C in a blood- or hemoglobin-containing medium. Long-term storage is best accomplished by lyophilization. Retrieval of viable cultures that have been lyophilized may be enhanced by suspending dried cells in a blood-broth mixture followed by culture on fresh blood agar media.

DIFFERENTIATION OF THE GENUS *BARTONELLA* FROM OTHER GENERA

The organism originally described as the cat scratch bacillus (*Afipia felis*) is distinct genotypically and phenotypically from *Bartonella* spp. It is ~90% related to *Bartonella* based on 16S rRNA gene sequence similarity. See Table BXII.α.119 for additional characteristics useful in differentiating the *Bartonella* spp. from the genus *Afipia*. Another organism found in the blood of cats and similarly confused in the past with *Bartonella*, *Haemobartonella felis*, can now be clearly identified using a set of *H. felis*-specific primers that selectively amplify a 1316-bp DNA fragment of the 16S rRNA gene of *H. felis* (Messick et al., 1998).

TABLE BXII.α.119. Differential characteristics of the *Bartonella* species, *Aftipia felis*, and *Brucella melitensis*^a

Characteristic	<i>B. bacilliformis</i>	<i>B. alsatica</i>	<i>B. clarridgeiae</i>	<i>B. doshiae</i>	<i>B. elizabethae</i>	<i>B. grahamii</i>	<i>B. henselae</i>	<i>B. koehlerae</i>	<i>B. peromysci</i>
Optimal temp, °C	25–30	35	35–37	35–37	35–37	35–37	35–37	35	20–28
Growth in nutrient broth	–	–	–	–	–	–	–	–	–
Growth on heart infusion agar with X factor	–	–	–	–	+	–	–	–	–
Hemolysis	–	–	–	–	v	–	–	–	–
Growth in <10 d	+	–	+	v	+	+	v	–	+
Oxidase	–	–	–	–	–	–	–	–	–
Catalase	+	–	–	–	–	–	v	–	–
Nitrate reduction	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–
Glucose oxidation or fermentation	–	–	–	–	–	–	–	–	–
Voges-Proskauer	–	–	–	+	–	+	–	–	–
<i>p</i> -Nitrophenyl-β-D-galactopyranosidase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl-α-D-galactopyranosidase	–	–	–	–	–	–	–	–	–
bis <i>p</i> -Nitrophenyl-phosphatase	+	–	–	–	+	–	+	+	–
<i>p</i> -Nitrophenyl-N-acetyl-β-D-glucosaminidase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl-α-D-glucopyranosidase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl-β-D-glucopyranosidase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl phosphatase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl-α-L-fucopyranosidase	–	–	–	–	–	–	–	–	–
<i>p</i> -Nitrophenyl-α-D-mannopyranosidase	–	–	–	–	–	–	–	–	–
L-Leucine-β-naphthyl-amidase	+	+	–	+	+	+	+	+	–
DL-Methionine-β-naphthyl-amidase	+	+	–	–	+	–	+	+	–
L-Lysine-β-naphthylamidase (alkaline)	+	+	–	–	+	–	+	+	–
L-Lysine-β-naphthyl-amidase (acidic)	+	+	–	–	w	–	+	–	–
Glycylglycine-β-naphthylamidase	+	+	–	–	+	–	+	+	–
Glycine-β-naphthylamidase	+	+	+	–	+	–	+	+	–
L-Proline-β-naphthylamidase	–	+	+	–	–	+	+	–	–
L-Arginine-β-naphthyl-amidase	+	+	+	–	+	–	+	+	–
L-Pyrrolindonyl-β-naphthylamidase	–	–	–	–	–	–	–	–	–
L-Tryptophan-β-naphthylamidase	+	+	–	–	+	–	+	+	–
3-Indoxyl phosphatase	–	–	–	–	–	–	–	–	–
Flagella	+	–	+	–	–	–	–	–	–
Twitching motility	–	–	–	–	–	–	+	–	–
Major cellular fatty acids:									
C _{16:0}	+	–	+	–	+	–	+	–	–
C _{16:1 ω7c}	+	–	–	–	–	–	–	–	–
C _{17:0}	–	–	–	–	+	–	–	–	–
C _{18:0}	–	–	+	–	–	–	+	–	–
C _{18:1 ω7c}	+	–	+	–	+	–	+	–	–
C _{19:0 cyclo}	–	–	–	–	–	–	–	–	–
C _{Bt-19:1}	–	–	–	–	–	–	–	–	–
Reactivity with fluorescent antibody to:									
<i>B. bacilliformis</i>	+	–	–	–	–	–	–	–	–
<i>B. doshiae</i>	–	–	–	+	–	w	–	–	–
<i>B. elizabethae</i>	–	–	–	–	+	–	–	–	–
<i>B. grahamii</i>	–	–	–	w	w	+	w	–	–
<i>B. henselae</i>	–	–	–	–	–	–	+	–	–
<i>B. quintana</i>	–	–	–	–	–	–	–	–	–
<i>B. taylorii</i>	–	–	–	w	–	–	w	–	–
<i>B. vinsonii</i>	–	–	–	–	–	–	–	–	–

^aSymbols: +, positive; –, negative; w, weakly positive; v, variable (only some isolates have the characteristic).

(continued)

TAXONOMIC COMMENTS

Bartonella is the only genus of the family *Bartonellaceae* within the *Alphaproteobacteria*. *B. bacilliformis* was the original and only member of the genus until 1993. The genus *Rochalimaea* was then united with the genus *Bartonella* in the family *Bartonellaceae* based on a proposal by Brenner et al. (1993). 16S rRNA gene sequence data and DNA hybridization data revealed high levels of relat-

edness between *Bartonella bacilliformis* and the four *Rochalimaea* species, indicating that these species were members of a single genus. The name *Bartonella* was retained as the genus name since it had nomenclatural priority over the name *Rochalimaea*. More recently, the genera of *Grahamella* and *Bartonella* were merged, and at present, 16 species/subspecies belong to the genus. The former *Rochalimaea* and, subsequently, *Grahamella* spp. were uni-

TABLE BXII.α.119. (cont.)

Characteristic	<i>B. quintana</i>	<i>B. talpae</i>	<i>B. taylorii</i>	<i>B. tribocorum</i>	<i>B. vinsonii</i> subsp. <i>vinsonii</i>	<i>B. vinsonii</i> subsp. <i>arupensis</i>	<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	<i>Afipia felis</i>	<i>Brucella melitensis</i>
Optimal temp, °C	35–37		35–37	35	35–37	35–37	35–37	25–30	36–38
Growth in nutrient broth	—	—	—	—	—	—	—	+	—
Growth on heart infusion agar with X factor	v				v	+	—	+	+
Hemolysis	—	—			—	—	—	—	—
Growth in <10 d	+	+	+		+	+	+	+	+
Oxidase	v			—	v	—	—	+	+
Catalase	—			—	v	—	v	—	+
Nitrate reduction	—			—	—	—	—	+	+
Indole	—			—	—	—	—	—	—
Urease	—			—	—	—	—	+	+
Glucose oxidation or fermentation	—	—	—	—	—	—	—	—	+
Voges-Proskauer	—		+	—	—				—
<i>p</i> -Nitrophenyl-β-D-galactopyranosidase	—			—	—	—	—		—
<i>p</i> -Nitrophenyl-α-D-galactopyranosidase	—		—	—	—	—	—		—
bis <i>p</i> -Nitrophenyl-phosphatase	v			—	+	+	+		—
<i>p</i> -Nitrophenyl- <i>N</i> -acetyl-β-D-glucosaminidase	—	—	—	—	—	—	—		+
<i>p</i> -Nitrophenyl-α-D-glucopyranosidase	—		—	—	—	—	—		+
<i>p</i> -Nitrophenyl-β-D-glucopyranosidase	—		—	—	—	—	—		—
<i>p</i> -Nitrophenyl phosphatase	—	—	—	—	—	—	—		+
<i>p</i> -Nitrophenyl-α-L-fucopyranosidase	—		—	—	—	—	—		—
<i>p</i> -Nitrophenyl-α-D-mannopyranosidase	—		—	—	—	—	—		—
L-Leucine-β-naphthylamidase	+		+	+	+	+	+		—
D,L-Methionine-β-naphthylamidase	+			+	+	w	+		+
L-Lysine-β-naphthylamidase (alkaline)	+			+	+	+	+		+
L-Lysine-β-naphthylamidase (acidic)	—			+	—	—	+		—
Glycylglycine-β-naphthylamidase	+			+	+	+	+		+
Glycine-β-naphthylamidase	+			+	+	+	+		+
L-Proline-β-naphthylamidase	+		+	—	v	—	+		+
L-Arginine-β-naphthylamidase	+			+	+	+	+		+
L-Pyrrolindonyl-β-naphthylamidase	—		—	—	—	—	—		—
L-Tryptophan-β-naphthylamidase	+			+	+	+	+		—
3-Indoxyl phosphatase	—			—	—	—	—		—
Flagella	—	—		—	—	—	—	+	—
Twitching motility	+				—	—	—		
Major cellular fatty acids:									
C _{16:0}	+				+	+	+		+
C _{16:1 ω7c}									
C _{17:0}					+	+			+
C _{18:0}	+				+	+	+		+
C _{18:1 ω7c}	+				+	+	+	+	
C _{19:0 cyclo}								+	+
C _{Br-19:1}								+	
Reactivity with fluorescent antibody to:									
<i>B. bacilliformis</i>	—		—		—				
<i>B. doshiae</i>	w		w		w				
<i>B. elizabethae</i>	—		—		—				
<i>B. grahamii</i>	w		w		w				
<i>B. henselae</i>	v		—		v	—		—	
<i>B. quintana</i>	+		—		—	—			
<i>B. taylorii</i>	w		+		w				
<i>B. vinsonii</i>	w		—		+				

^aSymbols: +, positive; —, negative; w, weakly positive; v, variable (only some isolates have the characteristic).

fied as *Bartonella* based on DNA–DNA hybridization data showing that they were not as closely related as previously thought to members of the order *Rickettsiales* (Brenner et al., 1993).

There are a number of unclassified isolates, mostly recovered

from rodents, presumed to be *Bartonella* spp. based on 16S rRNA gene sequence data. Further growth in the number of species and subspecies in the genus is thus expected to occur in the future.

ACKNOWLEDGMENTS

This description of the genus *Bartonella* contains information presented by Miodrag Ristic and Julius P. Kreier in the first edition of this *Manual*.

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Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int. J. Syst. Bacteriol. 43: 777–786.

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List of species of the genus *Bartonella*

- Bartonella bacilliformis*** (Strong, Tyzzer, Brues, Sellards and Gastiaturú 1913) Strong, Tyzzer and Sellards 1915, 808^{AL} (*Bartonia bacilliformis* Strong, Tyzzer, Brues, Sellards and Gastiaturú 1913, 1715.)
ba.cil.li.for' mis. L. dim. n. *bacillus* a small staff, rodlet; L. n. *forma* shape, form; M.L. adj. *bacilliformis* rod-shaped.

Displays characteristics of the genus *Bartonella*. Optimal growth occurs at 25–28°C on enriched media. Cells have 1–10 polar flagella; some may have subpolar or lateral flagella. Etiologic agent of Carrion's disease (Oroya fever, Verruga Peruana). Other characteristics useful in identification are shown in Table BXII.α.119.

Genome size is 4×10^8 Da.

The mol% G + C of the DNA is: 39 (T_m).

Type strain: ATCC 35685.

GenBank accession number (16S rRNA): Z11683.

Additional Remarks: The neotype strain ATCC 35685 was proposed by Brenner et al. (1991b).

- Bartonella alsatica*** Heller, Kubina, Mariet, Riegel, Desacour, Dehio, LaMarque, Kasten, Boulouis, Mounteil, Chomel and Piémont 1999, 287^{VP}
al.sa'ti.ca. L. adj. *alsatica* from Alsace, the region in eastern France near the Rhine River where wild rabbits, from which strains of the species were isolated and identified, were trapped.

Displays characteristics of the genus *Bartonella*. Colonies grown on blood agar appeared after 10 d as small, white, smooth, regular colonies (diameter ~1 mm). Additional characteristics useful in Identification are shown in Table BXII.α.119.

The mol% G + C of the DNA is: 37 (capillary electrophoresis).

Type strain: IBS 382, CIP 105477.

GenBank accession number (16S rRNA): AJ002139.

- Bartonella birtlesii*** Bermond, Heller, Barrat, Delacour, Dehio, Alliot, Monteil, Chomel, Boulouis and Piémont 2000, 1978^{VP}
birt.les'i.i. M.L. gen. n. *birtlesii* of Richard J. Birtles, whose studies have contributed to an improved understanding of the taxonomy of the genus.

The mol% G + C of the DNA is: not determined.

Type strain: IBS 325, CIP 106294, CCUG 44360.

GenBank accession number (16S rRNA): AF204274.

- Bartonella clarridgeiae*** Lawson and Collins 1996b, 836^{VP} (Effective publication: Lawson and Collins 1996a, 71.)
clar.ridge'i.a.e. M.L. fem. adj. *clarridgeiae* named in honor of Jill E. Clarridge III, the microbiologist who first isolated the organism, in Houston, Texas.

Displays characteristics of the genus *Bartonella*. Possesses polar flagella. Additional characteristics useful in identification are shown in Table BXII.α.119.

The mol% G + C of the DNA is: not determined.

Type strain: Houston-2 cat, ATCC 51734.

GenBank accession number (16S rRNA): X89208.

- Bartonella doshiae*** Birtles, Harrison, Saunders and Molyneux 1995, 7^{VP}
do'shi.ae. M.L. gen. n. *doshiae* named in honor of Nivedita Doshi, who was technically responsible for work with *Legionella* and *Bartonella* at the Central Public Health Laboratory in London.

Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119.

The mol% G + C of the DNA is: 41 (T_m).

Type strain: R18, ATCC 700133, NCTC 12862.

GenBank accession number (16S rRNA): Z31351.

- Bartonella elizabethae*** (Daly, Worthington, Brenner, Moss, Hollis, Weyant, Steigerwalt, Weaver, Daneshvar and O'Connor 1993) Brenner, O'Connor, Winkler and Steigerwalt 1993, 785^{VP} (*Rochalimaea elizabethae* Daly, Worthington, Brenner, Moss, Hollis, Weyant, Steigerwalt, Weaver, Daneshvar and O'Connor 1993, 880.)
e.liz'a.beth.a.e. M.L. fem. adj. *elizabethae* named after St. Elizabeth's Hospital in Brighton, Massachusetts, where the organism was isolated.

Displays characteristics of the genus *Bartonella*. There is incomplete hemolysis on rabbit blood agar. Additional characteristics useful in identification are shown in Table BXII.α.119. Etiologic agent of endocarditis and neuroretinitis.

The mol% G + C of the DNA is: 41 (T_m).

Type strain: F9251, B91-002005, ATCC 49927.

GenBank accession number (16S rRNA): L01260.

- Bartonella grahamii*** Birtles, Harrison, Saunders and Molyneux 1995, 7^{VP}
gra.ham'i.i. M.L. gen. n. *grahamii* of Graham, named in honor of G.S. Graham-Smith who observed the organisms subsequently named *Grahamella* in the blood of moles.

Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119.

The mol% G + C of the DNA is: 40 (T_m).

Type strain: V2, ATCC 700132, NCTC 12860.

GenBank accession number (16S rRNA): Z31349.

8. **Bartonella henselae** (Regnery, Anderson, Clarridge, Rodriguez-Barradas and Jones 1992) Brenner, O'Connor, Winkler and Steigerwalt 1993, 785^{VP} (*Rochalimaea henselae* Regnery, Anderson, Clarridge, Rodriguez-Barradas and Jones 1992, 272.)
hen'sel.a.e. M.L. gen. n. *henselae* named in honor of Diane M. Hensel, who isolated many of the original strains detected in bacteremic patients from Oklahoma.
Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119. Etiologic agent of septicemia in immunocompromised hosts, endocarditis, bacillary angiomatosis, peliosis hepatis, cat scratch disease, and HIV-associated neurological syndromes.
The mol% G + C of the DNA is: 41 (T_m).
Type strain: Houston-1, G5436, ATCC 49882.
GenBank accession number (16S rRNA): M73229.
9. **Bartonella koehlerae** Droz, Chi, Horn, Steigerwalt, Whitney and Brenner 2000, 423^{VP} (Effective publication: Droz, Chi, Horn, Steigerwalt, Whitney and Brenner 1999, 1122.)
koeh'ler. ae. M.L. fem. adj. *koehlerae* named in honor of Jane E. Koehler, who was the first to isolate *Bartonella* species from bacillary angiomatosis lesions and whose studies of *B. quintana* and *B. henselae* isolates from human immunodeficiency virus-infected patients have contributed to an improved understanding of *Bartonella*-associated disease in humans.
Displays characteristics of the genus *Bartonella*. Growth is optimal on chocolate agar, and primary colonies are observed after 14 d of incubation at 35°C in a CO₂-enriched environment. Additional characteristics useful in identification are shown in Table BXII.α.119.
The mol% G + C of the DNA is: not determined.
Type strain: C-29, ATCC 700693.
GenBank accession number (16S rRNA): AF076237.
10. **Bartonella peromysci** (Ristic and Kreier 1984b) Birtles, Harrison, Saunders and Molyneux 1995, 7^{VP} (*Grahamella peromysci* (ex Tyzzer 1942) Ristic and Kreier 1984b, 719.)
pe.ro.mys'ci. M.L. gen. n. *peromysci*, of *Peromyscus* a genus of mice.
Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119. Distinguished from *B. talpae* by the host specificity.
The mol% G + C of the DNA is: unknown.
Type strain: No type strain available.
11. **Bartonella quintana** (Schmincke 1917) Brenner, O'Connor, Winkler and Steigerwalt 1993, 784^{VP} (*Rochalimaea quintana* (Schmincke 1917) Krieg 1961, 163; *Rickettsia quintana* Schmincke 1917, 961.)
quin.ta'na. M.L. fem. adj. *quintana* fifth, referring to 5-day fever and the clinical disease produced by the species.
Displays characteristics of the genus *Bartonella*. Etiologic agent of trench fever, bacillary angiomatosis, and septicemia. Additional characteristics useful in identification are shown in Table BXII.α.119.
The mol% G + C of the DNA is: 40 (T_m).
Type strain: ATCC VR-358.
GenBank accession number (16S rRNA): M11927, M73228.
12. **Bartonella talpae** (Ristic and Kreier 1984b) Birtles, Harrison, Saunders and Molyneux 1995, 7^{VP} (*Grahamella talpae* (ex Brumpt 1911) Ristic and Kreier 1984b, 719.)
tal'pae. M.L. gen. n. *talpae* of *Talpa*; M.L. fem. n. a genus of moles.
Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119. Distinguished from *B. peromysci* by the host specificity.
The mol% G + C of the DNA is: unknown.
Type strain: No type strain is available.
13. **Bartonella taylorii** Birtles, Harrison, Saunders and Molyneux 1995, 7^{VP}
tay.lor'i.i. M.L. gen. n. *taylorii* of Taylor, named in honor of A.G. Taylor who led various microbiologic studies at the Central Public Health Laboratory in London.
Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119.
The mol% G + C of the DNA is: 41 (T_m).
Type strain: M6, NCTC 12861.
GenBank accession number (16S rRNA): Z31350.
14. **Bartonella tribocorum** Heller, Riegel, Hansmann, Delacour, Bermond, Dehio, Lamarque, Monteil, Chomel and Piémont 1998, 1338^{VP}
tri.bo.co'rum. L. n. gen. pl. *Triboci* the tribes, mentioned by Caesar (51 BC) in his *Commentarii de Bello Gallico*, which were living in the region near the Rhine River in eastern France. Wild rats, from which two strains of the species were isolated, were trapped there.
Displays characteristics of the genus *Bartonella*. Colonies grown on blood agar appear as small, white, smooth, regular colonies (~1 mm diameter) after 10 d. Electron microscopic examination shows small bacilli without flagella, approximately 1–2 × 0.5 µm. Additional characteristics useful in identification are shown in Table BXII.α.119.
The mol% G + C of the DNA is: 38 (capillary electrophoresis).
Type strain: IBS 506, CIP 105476.
GenBank accession number (16S rRNA): AJ003070.
15. **Bartonella vinsonii** (Weiss and Dasch 1982) Brenner, O'Connor, Winkler and Steigerwalt 1993, 785^{VP} emend. Kordick, Swaminathan, Greene, Wilson, Whitney, O'Connor, Hollis, Matar, Steigerwalt, Malcolm, Hayes, Hadfield, Breitschwerdt and Brenner 1996, 708 (*Rochalimaea vinsonii* Weiss and Dasch 1982, 313.)
vin.so'ni.i. N.L. gen. n. *vinsonii* named in honor of J. William Vinson who, with Henry S. Fuller, originally demonstrated that *Bartonella vinsonii* subsp. *vinsonii* (*Rochalimaea vinsonii*) could be grown on blood agar.
The mol% G + C of the DNA is: See subspecies data below.
Type strain: See subspecies below.
- a. **Bartonella vinsonii subsp. vinsonii** (Weiss and Dasch 1982) Brenner, O'Connor, Winkler and Steigerwalt 1993, 785^{VP} emend. Kordick, Swaminathan, Greene, Wilson, Whitney, O'Connor, Hollis, Matar, Steigerwalt, Malcolm, Hayes, Hadfield, Breitschwerdt and Brenner 1996, 708 (*Bartonella vinsonii* (Weiss and Dasch 1982) Brenner, O'Connor, Winkler and Steigerwalt 1993, 785; *Rochalimaea vinsonii* Weiss and Dasch 1982, 313.)

Displays characteristics of the genus *Bartonella*. Additional characteristics useful in identification are shown in Table BXII.α.119. Vole is the animal host (also known as the “Canadian vole agent”).

The mol% G + C of the DNA is: 41 (T_m) (Daly et al., 1993).

Type strain: ATCC VR-152.

GenBank accession number (16S rRNA): L01259, Z31352.

- b. *Bartonella vinsonii* subsp. *arupensis* Welch, Carroll, Hofmeister, Persing, Robison, Steigerwalt and Brenner 2000, 3^{VP} (Effective publication: Welch, Carroll, Hofmeister, Persing, Robison, Steigerwalt and Brenner 1999, 2601.) *a.rup.en'sis*. N.L. fem. adj. *arupensis* coming from ARUP (Associated Regional and University Pathologists, Inc.) in Salt Lake City, Utah, the laboratory where the type strain was initially characterized.

Exhibits characteristics of the species *Bartonella vinsonii*. Grows on heart infusion agar in the presence of X factor. Other characteristics useful in identification are shown in Table BXII.α.119. Isolated from a human and mice (*Peromyscus leucopus*). Presumptively pathogenic for humans.

The mol% G + C of the DNA is: not determined.

Type strain: OK 94-513, ATCC 700727, isolated from a 62-year-old bacteremic man.

GenBank accession number (16S rRNA): U71322.

Additional Remarks: Other reference strain, UMB (Hofmeister et al., 1998, 413).

- c. *Bartonella vinsonii* subsp. *berkhoffii* Kordick, Swaminathan, Greene, Wilson, Whitney, O'Connor, Hollis, Matar, Steigerwalt, Malcolm, Hayes, Hadfield, Breitschwerdt and Brenner 1996, 908^{VP} *berk.hoff'i.i*. M.L. gen. n. *berkhoffii* named in honor of Herman A. Berkhoff, a veterinary microbiologist whose research contributed to the understanding of *Bartonella*

infections in domestic animals and recognition of this subspecies.

Exhibits characteristics of the species *Bartonella vinsonii*. Can be distinguished from *Bartonella vinsonii* subsp. *vinsonii* by DNA methods. 16S rRNA gene contains an insertion sequence designated II-I12 in the variable region V2. Other characteristics useful in identification are shown in Table BXII.α.119. Etiologic agent of canine endocarditis.

The mol% G + C of the DNA is: not determined.

Type strain: 93-CO1, ATCC 51672.

GenBank accession number (16S rRNA): L35052.

Species Incertae Sedis

1. *Wolbachia melophagi* (Nöller 1917) Philip 1956, 267^{AL} (Nöller 1917, 70.)*

me.lo.pha'gi. M.L. gen. n. *melophagi* of *Melophagus*, named after the genus of its natural host, *Melophagus ovinus*, a wingless fly commonly called sheep ked.

The characteristics are as described for the genus *Wolbachia* with the following additional characteristics. Coccoid cells 0.4–0.6 μm or short rods 0.3–1.0 μm. Grow on blood-glucose-bouillon agar and chicken embryo yolk sacs but not in cultured eucaryotic cells. Natural hosts are a wingless fly, *Melophagus ovinus*, and *Diptera* that infest goats, pigs, and horses. Grows extracellularly in the host insect's gut, where the wolbachiae form rows of short rods associated with the intestinal epithelium. Apparently nonpathogenic for hosts.

The mol% G + C of the DNA is: not determined.

Type strain: no strain isolated.

GenBank accession number (16S rRNA): X89110 (strain MO6).

*Editorial Note: Dumler et al. (2001) have indicated that 16S rDNA sequence analysis places *Wolbachia melophagi* in the genus *Bartonella*.

Family III. *Brucellaceae* Breed, Murray and Smith 1957, 394^{AL}

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Bru.cel.la'ce.ae. M.L. fem. n. *Brucella* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Brucellaceae* the *Brucella* family.

The family *Brucellaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA gene sequences; the family contains the genera *Brucella* (type genus), *Mycoplana*, and *Ochrobactrum*.

Chemoorganotrophs with an aerobic, respiratory metabolism. Family includes pathogens and soil organisms.

Type genus: **Brucella** Meyer and Shaw 1920, 173^{AL}

Genus I. Brucella Meyer and Shaw 1920, 173^{AL}

MICHAEL J. CORBEL AND MENACHEM BANAI

Bru. cel'la. L. dim. ending *-ella*; M.L. fem. n. *Brucella* named after Sir David Bruce, who first recognized the organism causing undulant (Malta) fever.

Cocci, coccobacilli, or short rods, 0.5–0.7 × 0.6–1.5 μm. Arranged singly and, less frequently, in pairs, short chains, or small groups. True capsules are not produced. Do not usually show true bipolar staining. Resting stages are not known. Gram neg-

ative. **Nonmotile**; do not produce flagella. **Aerobic**, possessing a respiratory type of metabolism and having a **cytochrome-based electron transport system** with oxygen or nitrate as the terminal electron acceptor. Nitrate reductase is produced. **Many strains**

require supplementary CO₂ for growth, especially on primary isolation. Colonies on serum-dextrose agar or other clear media are transparent, raised, convex, with an entire edge and a smooth, shiny surface. They appear a **pale honey color** by transmitted light. Smooth strains produce perosamine synthetase and a distinctive lipopolysaccharide (LPS). Nonsmooth variants of the smooth species occur, but there are also stable nonsmooth nomenclatures with a distinctive host range. Optimal temperature for growth 37°C. Growth occurs between 20 and 40°C. Optimal pH for growth 6.6–7.4. **Catalase positive**. Usually oxidase positive, but negative strains occur. Chemoorganotrophic. Most strains require complex media containing several amino acids, thiamin, nicotinamide, iron, and magnesium ions; some strains may be induced to grow on minimal media containing an ammonium salt as the sole nitrogen source. **Growth is improved by serum or blood, but hemin (X-factor) and nicotinamide adenine dinucleotide (NAD: V-factor) are not essential**. **Acid production does not occur from carbohydrates in conventional media**, except for *B. neotomae*. Do not produce indole. **Do not liquefy gelatin** or inspissated serum. Do not lyse erythrocytes. Do not produce **acetyl methyl carbinol** (Voges-Proskauer test.) **The methyl red test is negative**. Intracellular parasites, transmissible to a wide range of animal species including man. The genome typically comprises two circular chromosomes, but a single large chromosome is present in *B. suis* biovar 3.

The genus is essentially genetically **monospecific**, nomenclatures and biovars reflecting deletions, insertions, or rearrangements in a largely conserved genome. The 16S rRNA gene sequence data, distinctive lipid composition, shared proteins and sensitivity to trifolitoxin indicate affiliation to the order *Rhizobiales* in the *Alphaproteobacteria*. The closest relationships are to the genus *Ochrobactrum* and to a lesser extent to the genus *Mycoplana*, and the three genera comprise the known membership of the family *Brucellaceae*.

The mol% G + C of the DNA is: 57.9–59.

Type species: *Brucella melitensis* (Hughes 1893) Meyer and Shaw 1920, 179 (*Streptococcus melitensis* Hughes 1893, 235.)

FURTHER DESCRIPTIVE INFORMATION

Cell morphology When grown in nutritionally adequate liquid or solid media such as serum-dextrose broth (SDB)¹ or serum-dextrose agar (SDA)², *Brucella* cells are coccoid, coccobacilli, or short rods with slightly convex sides and rounded ends. Freshly isolated strains tend to be more coccoid than laboratory-adapted cultures; this is also true of organisms growing *in vivo*. In general, the morphology of *Brucella* strains is constant and pleomorphic forms are rare, except in old cultures growing under adverse conditions.

1. Serum-dextrose broth (SDB): tryptone-soya broth (Oxoid), 30 g; distilled water, 1000 ml; sterile horse serum (inactivated at 56°C for 30 min), 50 or 100 ml; D-glucose (25%, w/v, solution autoclaved at 105°C for 20 min), 40 ml. The medium is prepared by dissolving the tryptone-soya broth powder in the water and autoclaving at 115°C for 15 min. After cooling, the sterile horse serum and glucose are added aseptically. Note: Any good quality peptone medium such as Tryptose broth (Difco), or *Brucella* broth (Gibco) may be used as alternatives to tryptone-soya broth. Serum-dextrose agar (SDA): blood agar base No. 2 (Oxoid) 40 g; distilled water, 1000 ml; sterile horse serum (inactivated at 56°C for 30 min), 50 or 100 ml; D-glucose ((25% w/v) solution autoclaved at 105°C for 20 min), 40 ml. The blood agar base is dissolved in the water with the aid of gentle heating and then autoclaved at 121°C for 15 min. After cooling, the horse serum and glucose are added aseptically and plates or slopes poured immediately. Note: Tryptose agar (Difco) or *Brucella* agar (Gibco) are satisfactory alternative basal media.

Brucella cells stain readily by conventional methods. Although not truly acid fast, they do tend to resist decolorization by weak acids and thus stain red by Macchiavello's stain or by the modified Ziehl-Neelsen technique used by Stamp et al. (1950). They are usually stained red by the modified Köster method (Christoffer-son and Ottosen, 1941), but *B. ovis* is an exception. True capsules do not occur although capsule-like structures have been reported.

Fine structure The ultrastructure of the *Brucella* cell is broadly similar to that of other Gram-negative bacteria but shows a number of significant differences from that of cells of the *Enterobacteriaceae*, as typified by *Escherichia coli* (De Petris et al., 1964; Dubray, 1972, 1976; Dubray and Plommet, 1976).

The *Brucella* cell wall is composed of an outer membrane comprising an external layer of lipopolysaccharide (LPS), a range of outer membrane proteins including some with porin activity, lipoproteins, and phospholipids. In negatively stained thin sections, this layer appears about 9 nm thick (Dubray, 1976). It is the location of the major surface antigens in both smooth and nonsmooth cells. It is supported by an electron-dense inner layer 3.5 nm thick corresponding to cross-linked muramic-acid-containing peptidoglycan. Some of the outer membrane proteins extend through this layer to the periplasmic region, which appears as a zone of low electron density 3–6 nm thick in smooth-phase cells but up to 30 nm thick in nonsmooth cells (Dubray and Plommet, 1976). As in other Gram-negative bacteria, it is the site of periplasmic enzymes involved in cell wall biosynthesis and various metabolic functions.

The cytoplasmic inner membrane has a typical three-layered lipoprotein structure (De Petris et al., 1964; Dubray and Plommet, 1976). Granular aggregations adjacent to the cytoplasmic membrane mark the location of polyribosomal complexes (Dubray, 1972, 1976).

The *Brucella* cytoplasm is homogeneous and is interspersed with small vacuoles and polysaccharide-containing granules (Dubray, 1972). The nuclear apparatus comprises an osmiophobic mass intersected by osmiophilic filamentous structures (De Petris et al., 1964; Peschkov and Feodorov, 1978).

Cell wall composition The detailed chemical structure of the *Brucella* cell wall has not been fully determined. Gross analyses have indicated that the cell wall accounts for about 21% of the total bacterial dry weight in smooth cultures and 14% in nonsmooth strains. The walls of smooth *Brucella* cells contain approximately 37% protein, 14% carbohydrate, 18% lipid, 0.4% muramic acid, and 0.1% 2-keto-3-deoxyoctulosonic acid (KDO). For nonsmooth *Brucella* cells walls the corresponding values are approximately 47.5% protein, 13% carbohydrate, 17% lipid, 0.4% muramic acid, and 0.1% KDO (Kreutzer et al., 1977).

The external layer of the outer membrane comprises mainly LPS interspersed with a variety of proteins and lipids. The LPS of smooth *Brucella* cell walls is unusual in that it partitions into the phenol layer on phenol-water extraction (Baker and Wilson, 1965). The LPS of the nonsmooth strains is extremely hydrophobic and cannot be extracted with phenol-water but is soluble in phenol-hexane-chloroform (Moreno et al., 1979).

The LPS of both forms consists of a lipid A containing both 2,3-diamino-2,3-dideoxyglucose and 2-amino-2-deoxyglucose in the glycosyl backbone, a feature of some other members of the *Proteobacteria* (Weckesser and Mayer, 1988). Both amide- and ester-linked fatty acids are attached to the aminoglycosyl skeleton.

The amide-linked acids include 3-O-C_(16:0) 12:0 (25%), 3-O-C_(16:0) 13:0 (4%), 3-O-C_(16:0) 14:0 (64%), and 3-O-C_(18:0) 14:0 (7%) as diesters with C_{16:0} 3OH as the unsubstituted fatty acid. The ester-linked acids comprise C_{16:0}, C_{16:0} 3OH, C_{18:0} 3OH, and C_{18:0} acids, which account for 37%, 12.5%, 3.5%, and 4.5%, respectively, of the total fatty acids. Lactobacillic and unsaturated fatty acids are absent from the lipid A of *Brucella* although represented in many other lipid components (Cherwonogrodzky et al., 1990).

The lipid A is linked through KDO to the core polysaccharide composed of D-glucose, D-mannose, and 6-amino 6-deoxyglucose (quinovosamine) (Bowser et al., 1974). Heptose is absent from *Brucella* LPS. In the case of some rough strains, a short homopolymer of 4,6-dideoxy-4-formamido-D-mannose (*N*-formyl-D-perosamine) may be attached to the core polysaccharides (Perry and Bundle, 1990), but in many strains this is absent and some may also be deficient in quinovosamine (Moreno et al., 1984). D-Glucose and D-mannose, and possibly quinovosamine, are components of the major epitope of nonsmooth *Brucella* strains.

In the case of smooth strains, the LPS carries an O chain linked to the core polysaccharide. The O chain comprises a homopolymer of about 100 residues of *N*-formyl-D-perosamine. In the case of A-dominant *B. abortus* LPS, the O chain consists of a majority of glucose residues linked alpha-1,2, but with a very small proportion linked alpha-1,3 (Caroff et al., 1984a, b; Bundle et al., 1987).

In the case of M-dominant *B. melitensis* LPS, the O chains consist of unbranched linear polymers of pentasaccharide units, comprising four residues linked alpha-1,3. The difference in linkage produces penta- or hexasaccharide units with different preferred conformations. The common presence of nonterminal tetrasaccharide units of alpha-1,2 linked *N*-formyl-D-perosamine explains the crossreactivity observed between the LPS of all smooth *Brucella* strains (C epitope) (Cloeckaert et al., 1998).

The A- and M-specific epitopes are actually present as minority structures in both types of LPS. In the case of strains typified by *B. abortus* biovar 1, the O chains contain predominantly A epitopes with a very small proportion of M epitope, attributable to the few alpha-1,3 linked residues. In strains typified by *B. melitensis* biovar 1, the reverse situation applies. It should be noted that strains of *B. abortus*, *B. melitensis*, or *B. suis* can be A-, M-, or A- and M-antigen positive (see Table BXII.α.120 and Dubray and Limet, 1987). Strains that are both A- and M-antigen positive

synthesize LPS with O chains that contain both A and M structural features in relatively high proportion (Perry and Bundle, 1990).

N-Acyated-D-perosamine also occurs in the O chains of the LPS complexes of *E. coli* 0157, *Escherichia hermannii*, *Salmonella* O30 (Bundle et al., 1987), *Stenotrophomonas maltophilia* strain 555, *Vibrio cholerae* (Redmond, 1979; Kenne et al., 1982), and *Yersinia enterocolitica* 09 (Caroff et al., 1984b), all of which crossreact serologically with smooth *Brucella* strains (Corbel, 1985). Crossreactions also occur between *Brucella* and *Francisella tularensis* (Francis and Evans, 1926; Ohara et al., 1974), although the basis of this has yet to be determined.

The outer membrane proteins include the 88–94-kDa high molecular weight group 1, the 43 kDa and 36–38-kDa porin proteins of group 2 (Douglas et al., 1984), and the 25–27-kDa proteins of group 3, as well as minor proteins of 15–31 kDa. A lipoprotein of 8 kDa is also covalently linked to the peptidoglycan skeleton. These components are present in all the nomenclatures but with quantitative differences (Santos et al., 1984; Verstrete and Winter, 1984). In addition to the above, *B. melitensis* contains a 31 kDa outer-membrane protein and another of 30–40-kDa.

In all *Brucella* strains, the group 2 proteins are by far the most abundant. The genes for the 36-kDa porins (*omp2a* and *omp2b*) have been cloned, and the peptide sequences determined (Ficht et al., 1990). The function of the group 3 proteins, formerly assumed to be analogues of Omp A, has yet to be determined. The *omp2* genes and those encoding the Group 3 proteins demonstrate polymorphisms, which permit grouping into clusters corresponding approximately to nomenclatures and biovars (Cloeckaert et al., 1995). The 8 kDa lipoprotein resembles the Braun lipoprotein of *E. coli* in molecular weight, isoelectric point, and amino acid composition but differs from it in being surface exposed (Gomez-Miguel et al., 1987). The Omp 10, Omp 16, and Omp 19 outer membrane proteins are also reported to be lipoproteins (Tibor et al., 1999).

The *Brucella* outer membrane is unusual in being particularly rich in myristic, palmitic, and stearic acids, in containing moderate quantities of *cis*-vaccenic and arachidonic acids, low quantities of C₁₇ and C₁₉ cyclopropane fatty acids and no hydroxy fatty acids. This unusual fatty acid composition may contribute to hydrophobic interactions in the *Brucella* outer membrane and enhance its stability (Cherwonogrodzky et al., 1990).

TABLE BXII.α.120. Differentiation of the species and biovars of the genus *Brucella*^a

Characteristics	<i>B. melitensis</i> biovars			<i>B. abortus</i> biovars							<i>B. suis</i> biovars					<i>B.</i> <i>ovis</i>	<i>B.</i> <i>neotomae</i>	<i>B.</i> <i>canis</i>
	1	2	3	1	2	3 ^b	4	5	6 ^b	7	9	1	2	3	4	5		
CO ₂ requirement	–	–	–	[+]	[+]	[+]	[+]	–	–	–	–	–	–	–	–	–	+	–
H ₂ S production	–	–	–	+	+	+	+	–	[–]	[+]	+	+	–	–	–	–	+	–
Growth on media containing: ^c																		
Thionine	+	+	+	–	–	+	–	+	+	+	+	+	+	+	+	+	– ^d	+
Basic fuchsin	+	+	+	+	–	+	+	+	+	+	+	[–]	–	+	[–]	–	–	[–]
Agglutination with monospecific antisera:																		
A	–	+	+	+	+	–	–	–	+	+	–	+	+	+	+	–	+	–
M	+	–	+	–	–	–	+	+	–	+	+	–	–	–	+	–	–	–
R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+

^aSymbols: +, positive for all strains; [+], positive for most strains; [–], negative for most strains; –, negative for all strains.

^bFor more certain differentiation of biovar 3 and 6, thionine at 1:25,000 (w/v) is used; biovar 3 gives a positive growth response, biovar 6 is negative.

^cDye concentration, 1:50,000 (w/v).

^dGrowth will occur in the presence of thionine at a concentration of 1:150,000 (w/v).

The peptidoglycan framework of the cell wall comprises a glycan skeleton of D-glucosamine and muramic acid linked by short chains of alanine, glutamic acid, and alpha, epsilon-diaminopimelic acid. However, it also contains covalently linked proteins and lipid and is unusually resistant to lysozyme, even in the presence of detergents and chelating agents.

The lipid composition of *Brucella* cells has a number of distinctive features, which reinforce the taxonomic affiliation of the genus to the group of bacteria known as the alpha 2 subdivision of the *Proteobacteria*.

The bound lipids are mainly associated with the LPS, lipoprotein, and glycolipid fractions of the outer membrane. The free lipid fraction comprises mainly phospholipids and neutral lipids (Wober et al., 1964; Thiele and Kehr, 1969; Thiele and Schwinn, 1969). Phosphatidylcholine is the principal phospholipid, in contrast to most bacterial species (Thiele and Schwinn, 1973). It may also form an antigenic determinant (Casao et al., 1998). Its presence confers distinctive properties on the outer membrane and may account for its reduced susceptibility to phospholipases and lysozyme. Phosphatidylglycerol and diphosphatidylglycerol are also present as major lipids. Phosphatidyl ethanolamine, phosphatidyl serine, and cardiolipin are represented as minor components. The fatty acids associated with the phospholipid fraction are unusual in that lactobacillic (C_{19: cyclo}) acid, typical of Gram-positive but not Gram-negative species, is usually the major cyclopropane fatty acid, together with its metabolic precursor *cis*-vaccenic (C_{18:1 cyclo}) acid. *B. canis* is an exception, and has *cis*-vaccenic acid as the major fatty acid, with lactobacillic acid in only trace amounts. This largely accounts for the distinct position of *B. canis* vis-à-vis the other nomenclatures when fatty acid composition is used for taxonomic analysis (Tanaka et al., 1977).

The neutral lipids include unusual wax-like esters containing large quantities of myristic (C_{14:0}), palmitoleic (C_{16:1}), stearic (C_{18:0}) and *cis*-vaccenic (C_{18:1}) acids. Lactobacillic acid is absent from these compounds.

Other distinctive free lipid components of *Brucella* include ubiquinone Q-10 and ornithine-containing lipids (Thiele and Schwinn, 1973). The latter make up 32% of the total neutral lipid. They contain lactobacillic, *cis*-vaccenic, and palmitoleic acids in ester linkage and palmitic and stearic acids in amide linkage, but no hydroxy fatty acid. The function of the ornithine lipids is unknown but they are structural components of the outer membrane. It has been suggested that they are implicated in the attachment of *Brucella* cells to the surface of macrophages and lymphocytes (Cherwonogrodzky et al., 1990). Minor free lipid components include 1,3 and 1,2 diesters of glycerol and monoesters of ethylene glycol.

Cultural characteristics Most *Brucella* strains behave as slow-growing, fastidious organisms on primary isolation. Although laboratory-adapted strains may be induced to grow in synthetic media containing an ammonium salt as the sole nitrogen source, the majority of fresh isolates have complex nutritional requirements and grow poorly on ordinary nutrient media unless these are supplemented with blood, serum, or tissue extracts. Liver infusion agar was at one time widely employed for the cultivation of brucellae, but better-defined media of more consistent composition are now preferred.

For most purposes, SDA is the medium recommended and will support the growth of all species and most strains (Jones and Morgan, 1958). Tryptose agar (Difco), *Brucella* agar

(Gibco),³ and Tryptone-soya agar (Oxoid) or equivalent media will support the growth of most strains without serum supplementation and the growth of nearly all, if heated equine serum is added to a final concentration of 5–10% (v/v). The function of the serum is not simply nutritional but is also reported to neutralize inhibitors present in the peptone component of ordinary culture media, and may be replaced by other colloids, including Tween 40. Strains of *B. abortus* biovar 2 and some of biovar 4, and *B. ovis* are the most fastidious and grow best on media containing 10% (v/v) serum.

Potato infusion agar⁴ supports the growth of many *Brucella* strains and is often employed as the medium of choice for antigen or vaccine production, as it does not favor dissociation (Alton et al., 1988). For the most fastidious strains, it may be necessary to supplement this medium with horse serum to achieve satisfactory growth. Although wide variations occur between strains within nomenclature species, in general the most rapid growth and largest colony size are achieved by isolates of *B. suis* biovar 1 and 3 and *B. canis*. Growth is least vigorous for *B. ovis*, followed by many strains of *B. melitensis* biovar 1 and *B. suis* biovar 2. Strains of the other species and biovars usually occupy an intermediate position.

On primary isolation, colonies of any *Brucella* strain are rarely visible before 48 h. At this stage, colonies on SDA are usually 0.5–1.0 mm in diameter, raised, convex with a circular outline and an entire edge. In transmitted light, the colonies of smooth strains have a shiny surface and appear a clear pale yellow. In reflected light, the colonies have a smooth glistening surface but are slightly opalescent and bluish gray. The colonies of non-smooth strains are of similar size and shape to smooth colonies but vary considerably in color, consistency, and surface texture. They range from smooth-intermediate (SI) variants, which are morphologically indistinguishable from smooth (S) colonies but may differ in antigenic properties and phage susceptibility, through intermediate (I) forms to rough (R) and mucoid (M) variants.

R colonies are usually much less transparent than S colonies, with a more granular, dull surface, and range in color from matte white, yellowish white, or buff, to brown. Unlike S colonies, which are soft and easily emulsifiable to form stable suspensions in saline solutions, R colonies are often friable or viscous and difficult to detach cleanly from the agar surface. They will not form uniform suspensions in saline solutions but produce granular aggregates, threads, or clumps. M colonies are similar to R colonies in color and opacity but have a sticky glutinous texture.

The colonial variants of *Brucella* are best studied after four days' growth on glycerol-dextrose agar (GDA)⁵, under oblique illumination as described by Henry (1933). Differentiation of the various colonial types is greatly facilitated by staining with ammonium oxalate-crystal violet before examination in reflected

3. Albimi *Brucella* agar is no longer available, but an equivalent product is obtainable as *Brucella* agar from Gibco Laboratories, Grand Island, New York, NY 14072, U.S.A. High quality peptone based media from other suppliers are also satisfactory.

4. Potato infusion agar: Bacto potato infusion agar (Difco), 49.0 g; glycerol, 20.0 g; distilled water, 1000 ml. The glycerol is dissolved in the water and the dehydrated medium is suspended in this solution and heated to the boiling point until dissolved. The medium is sterilized at 121°C for 15 min. It should be prepared immediately before use.

5. Glycerol dextrose agar: to blood agar base No. 2 (Oxoid) or Tryptose agar (Difco) that has been autoclaved and cooled to 56°C, sterile solutions of glycerol and D-glucose are added to give final concentrations of 2% (w/v) and 1% (w/v), respectively.

light (White and Wilson, 1951). Under these conditions, S colonies appear pale yellow, R colonies are stained red with a coarse granular appearance, and other dissociated colonies are stained various shades of pink, purple, or blue. Apart from S, R, and M colonies, numerous transitional phases may occur in cultures undergoing dissociation to the nonsmooth state.

Nonsmooth variants may arise as a result of a genetic deletion leading to synthesis of an incomplete LPS structure. This can result in mutation or deletion of genes in the *rfb* operon, of which the perosamine synthetase gene is particularly critical (Godfroid et al., 1998). However, in some nonsmooth strains other changes may accompany the LPS modification and involve deeper structures within the cell wall (Dubray and Plommet, 1976; Kreutzer and Robertson, 1979).

Most *Brucella* strains grow moderately well on sheep blood agar but the colonial appearance is not distinctive. The organisms are nonhemolytic, but a greenish brown discoloration may develop around the colonies. This is most apparent in old cultures and is probably attributable to alkali production.

The more vigorous strains of *B. abortus*, *B. melitensis*, and *B. suis* will grow on MacConkey agar, producing small lactose-negative colonies. In general, the growth of the more fastidious *Brucella* strains is inhibited on media containing bile salts, tellurite, or selenite. Tolerance to synthetic dyes varies considerably between strains and is employed as the basis for differentiation of biovars (see below and Table BXII.α.120).

Nutrition and growth conditions Growth in simple nutrient liquid media is usually poor unless these are supplemented with blood, serum, or tissue extracts. Most strains will grow fairly well on unsupplemented, high-quality, enriched, peptone-based media such as *Brucella* broth (Gibco) or Tryptone broth (Difco).

Good growth is obtained in SDB or other media supplemented with serum. It is essential to maintain adequate aeration if satisfactory growth is to be obtained. After static incubation for 7 days at 37°C in SDB, smooth strains produce a slight to moderate uniform turbidity with a light, powdery deposit. Nonsmooth strains may produce a granular or slimy deposit, variable turbidity, and pellicle formation, sometimes accompanied by a "stalactite" appearance. Growth in static liquid media favors dissociation of S-phase cultures to nonsmooth forms. Vigorous aeration will prevent this if the medium remains adequately buffered near neutral pH.

In semisolid media, CO₂-requiring cultures of *B. abortus* and *B. ovis* produce a disk of growth a few millimeters below the surface of the medium. CO₂-independent *Brucella* species produce a uniform turbidity from the surface down to a depth of a few millimeters. There is no growth under the anaerobic conditions prevailing in static deep cultures.

The optimal growth temperature for all *Brucella* strains is 36–38°C, but growth of most strains will occur in the range 20–40°C. All strains lose viability at 56°C, although temperatures as high as 85°C may be necessary to ensure sterilization of dense suspensions (Swann et al., 1981).

Metabolism and metabolic pathways The organisms are aerobic, although many strains grow best under microaerophilic conditions. The electron transport of *B. abortus* (the only species closely studied in this regard) consists of a branched system involving cytochromes *a* + *a*₃, *b*, *c*, and *o*, flavoproteins, and ubiquinone. It is unusually resistant to respiratory inhibitors (Rest and Robertson, 1975). Energy-yielding processes are essentially oxidative, and *Brucella* cultures show little ability to acidify car-

bohydrate media in conventional tests. They have been reported to lack phosphofructokinase (Robertson and McCullough, 1968a, b), although this enzyme was reported in extracts of *B. suis* by Roessler et al. (1952). The inability to acidify carbohydrate media has also been attributed to inhibition by peptone constituents, and acidic reactions have been demonstrated in peptone-free media (Pickett and Nelson, 1955). *B. neotomae* is exceptional in that it is reported to produce acid (but not gas) from D-glucose, D-galactose, L-arabinose, and D-xylose in conventional peptone-water sugar media (Stoener and Lackman, 1957).

Glucose catabolism occurs via the hexose monophosphate pathway in conjunction with the tricarboxylic acid cycle (Robertson and McCullough, 1968a, b; Rest and Robertson, 1974). *meso*-Erythritol is metabolized by many *Brucella* strains in preference to glucose (Anderson and Smith, 1965), and D-erythritol-1-phosphate and other intermediates in the erythritol pathway will reduce the entire electron transport system (Rest and Robertson, 1975).

Although some laboratory-adapted strains will grow in minimal medium with an ammonium salt as the sole nitrogen source (McCullough and Dick, 1943), the nutritional requirements of *Brucella* cultures in general are complex. Multiple amino acids, thiamin, biotin, nicotinamide, iron, and magnesium ions are essential for growth; iron, manganese, and other trace elements exert a regulatory action. Iron acquisition is dependent on 2,3-dihydroxybenzoic acid siderophore production (Bellaire et al., 1999). Surplus iron is sequestered and stored by a bacterioferritin, which may also facilitate *in vivo* survival by blocking the catalytic role of iron in the formation of reactive oxygen intermediates (Denoel et al., 1995). The growth of many strains is stimulated by calcium pantothenate and *meso*-erythritol. Very few strains will grow with citrate as the sole carbon source.

Sulfur-containing amino acids and proteins are degraded and may be reduced to H₂S, but this varies with species and biovar. Indole is never produced from tryptophan or its proteins, and acetyl methyl carbinol is not produced from glucose (Voges-Proskauer reaction). Hydrolytic activity towards proteins in general is very limited and gelatin, inspissated serum, and litmus milk are not digested. *Brucella* strains either render the latter alkaline or produce no visible change.

The supplementary CO₂ required by strains of *B. abortus* and *B. ovis* for growth is used as a nutritional factor and not simply to lower oxygen tension or pH. It is incorporated directly into pyrimidines, glycine, and alanine (Newton et al., 1954).

The optimal pH for growth is between 6.6 and 7.4. Cultures die rapidly at pH 3.5 or below. Most *Brucella* strains produce alkali on protein or peptone-containing media, and this may act as a growth-limiting factor. Culture media should be adequately buffered near pH 7 for optimal growth. The optimal osmotic pressure is between 2 and 6 atmospheres, equivalent to between 0.05 and 0.15 M NaCl.

A wide range of carbohydrate and amino acid substrates is oxidized (McCullough and Beal, 1951). Manometric measurement of oxidation rates with selected substrates produces metabolic patterns that are characteristic of each species and some biovars (Meyer, 1961; Meyer and Cameron, 1961a, b). The oxidative metabolic patterns show a close correlation with the phage-sensitivity pattern and the preferred natural hosts of the species (Meyer and Morgan, 1962). As indicated in Table BXII.α.121, they are of primary importance in defining the species of *Brucella* and are of additional value in classifying the biovars of *B. suis*

TABLE BXII.α.121. Differentiation of the species of the genus *Brucella*^a

Characteristic	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i> biovar					<i>B. canis</i>	<i>B. neotomae</i>	<i>B. ovis</i>
			1	2	3	4	5			
<i>Lysis by Phage at RTD:</i>										
Tb	NL	L	NL	NL	NL	NL	NL	NL	PL	NL
Wb	NL	L	L	L	L	L	L	NL	L	NL
Fi	NL	L	PL	PL	PL	L	L	NL	L	NL
BK ₂	L	L	L	L	L	L	L	NL	L	NL
R/O	NL	PL	NL	NL	NL	NL	NL	NL	NL	L
R/C	NL	NL	NL	NL	NL	NL	NL	L	NL	L
<i>Oxidation of Substrate:</i>										
L-Alanine	+	+	d	—	d	—	—	d	d	d
L-Asparagine	+	+	—	d	—	—	+	—	+	+
L-Glutamic acid	+	+	—	d	d	d	+	+	+	+
L-Arabinose	—	+	+	+	—	—	—	d	+	—
D-Galactose	—	+	d	d	—	—	—	d	+	—
D-Ribose	—	+	+	+	+	+	+	+	d	—
L-Glucose	+	+	+	+	+	+	+	+	+	—
D-Xylose	—	d	+	+	+	+	+	—	—	—
L-Arginine	—	—	+	+	+	+	+	+	—	—
DL-Citrulline	—	—	+	+	+	+	+	+	—	—
DL-Ornithine	—	—	+	+	+	+	+	+	—	—
L-Lysine	—	—	+	—	+	+	+	+	—	—
meso-Erythritol	+	+	+	+	+	+	+	d	+	—
<i>Preferred host:</i>										
Cattle		+								
Desert wood rats									+	
Dogs								+		
Goats	+									
Hares				+						
Reindeer						+				
Rodents							+			
Sheep	+							+		+
Swine			+	+	+					

^aSymbols: +, positive; —, negative; d, doubtful; NL, no lysis; PL, partial lysis.

(Stableforth and Jones, 1963; Jones, 1967; Jones and Wundt, 1971).

Procedures for the determination of oxidative metabolic patterns by manometric methods are described by Morgan and Gower (1966), Corbel et al. (1979), and Alton et al., (1988). A nonquantitative technique, using thin layer chromatography to detect substrate utilization, has also been employed (Balke et al., 1977; Corbel et al., 1978). More recently, a simplified colorimetric method that uses chromogenic tetrazolium substrates, and has the advantages of speed and reduced hazard, has been developed (Broughton and Jahans, 1997). Irrespective of technique, the range of substrates used includes L-arabinose, D-glucose, D-ribose, D-xylose, meso-erythritol, L-alanine, L-asparagine, L-glutamic acid, L-arginine, L-lysine, DL-citrulline, and DL-ornithine. For some purposes, other substrates including adonitol, L-histidine, L-serine, D-amino acids, and urocanic acid may also be used.

All strains are catalase positive and superoxide dismutases are produced (Bricker et al., 1990). The species *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* are usually oxidase positive in tests with tetramethyl-*p*-phenylenediamine, but some strains are oxidase negative and this can be a useful epidemiological marker. Most *Brucella* strains produce nitrate reductase and reduce nitrates to nitrites. Nitrites may be further reduced (Zobell and Meyer, 1932). *B. canis*, *B. neotomae*, and *B. suis* strains normally show very strong urease activity. Most strains of *B. abortus* and *B. melitensis* also produce urease, but a few do not. This may be useful as an epidemiological marker. *B. ovis* does not usually hydrolyze urea, but some strains may do so on prolonged incubation.

Genetics

Genome composition The members of the genus form a close-knit group. The DNA base composition is in the range 57.9–59 mol% G + C. DNA–DNA hybridization studies have indicated that all nomenspecies show >90% similarity to the type strain (Hoyer and McCullough, 1968a, b; Verger et al., 1985; De Ley et al., 1987). This is consistent with the concept of a single species, and Verger et al. (1985) have proposed a system of classification in which all of the current nomenspecies would be classified as biovars of *B. melitensis*. However, recent advances in knowledge of the molecular genetics of *Brucella* have provided a clearer insight into the phylogenetic position of the genus and the evolution of the distinct types within it. Unexpectedly, the molecular genetic data have provided evidence in support of the classical differentiation into nomenspecies and biovars, which were defined on the basis of patterns of phenotypic characteristics that aligned with the preferred host specificity (Michaux-Charachon et al., 1997).

Restriction endonuclease analysis using low-cleavage-frequency enzymes such as *Xba*I or *Not*I, combined with pulsed field gel electrophoresis, produced patterns which were characteristic of each nomenspecies but showed little difference between biovars within these taxa (Allardet-Servent et al., 1988). This study also suggested a genome size of about 2.6×10^6 bp for *B. melitensis* 16M and *B. suis* 1330^T. However, development of a partial physical map of the *B. melitensis* biovar 1 strain 16M genome suggested an apparent size of about 3.3×10^6 bp (Allardet-Servent et al., 1991). An extension of this study led to the conclusion that the genome consisted of two chromosomes of 2.1

and 1.5×10^6 bp, respectively (Michaux et al., 1993). This was confirmed by insertion into each replicon of the *I-SceI* unique restriction site, which is a nonsymmetrical double-stranded sequence extending over 18 bp, followed by restriction fragment analysis with *PacI* and *SpeI* (Jumas-Bilak et al., 1995). Two rRNA operons and the gene for the DnaK heat shock protein are located on the large chromosome, and a third rRNA operon and the gene encoding the equivalent of the GroEL chaperonin are located on the small chromosome. As both replicons encode functions essential for survival and replication, they are clearly chromosomes rather than plasmids (Michaux et al., 1993).

Application of these methods to the full range of nomenclatures and biovars has produced a rather more complex picture, which has confirmed genomic differences between phenotypically distinguishable types (Michaux-Charachon et al., 1997; Jumas-Bilak et al., 1998). *B. melitensis* biovars 2 and 3 have a similar genomic structure to biovar 1. *B. abortus* strains have a large chromosome of about 2.1×10^6 bp and a small replicon of about 1.15×10^6 bp. However, in biovars 1–4 the small chromosome contains a 640×10^3 bp inversion, whereas in biovars 5–9 it resembles that of *B. melitensis*. The *B. suis* biovar 1 genome is grossly similar in size and structure to that of *B. melitensis* biovar 1, except that its small chromosome is about 50×10^3 bp longer. In contrast, *B. suis* biovars 2 and 4 each have a large chromosome of 1.85×10^6 bp and a small chromosome of 1.35×10^6 bp. *B. suis* biovar 3 appears to be unique in that it contains a single chromosome of 3.2×10^6 bp. This molecule incorporates the sequences present in the large and small chromosomes of the other types. The genome structure of *B. canis* is indistinguishable from that of *B. suis* biovar 1, reinforcing the view that it is a stable R mutant of that organism. On the other hand, the large chromosome of *B. neotomae* resembles that of *B. melitensis* biovar 1, whereas its small chromosome resembles that of *B. suis* biovar 1. The *B. ovis* genome is broadly similar to that of *B. melitensis* biovar 1 but contains 30 copies of the insertion sequence IS6501, compared with the 5–10 copies found in the other nomenclatures. The complete genome of *B. melitensis* 16M has been published (DeVecchio et al., 2002). Analysis of this genome has indicated the absence of Type I, II, and III secretion systems but the presence of Type IV and V secretion systems and genes encoding flagellin, hemolysin, and fusion proteins.

Within the genomes of all strains studied, numerous small insertions and deletions, ranging from $1\text{--}32 \times 10^3$ bp, and three *rrn* sites have been found. These may contribute to observed differences between nomenclatures and biovars (Mercier et al., 1996; Michaux-Charachon et al., 1997). Insertion and repeated sequences have been widely documented in *Brucella* strains (Halling and Zehr, 1990). These may influence the expression of specific genes. For example, the BCSP31 protein is not expressed by *B. ovis*, possibly because of the presence of multiple copies of a repeated sequence downstream of the gene. The insertion sequence IS6501 is universally present and has proved to be useful for identification of the genus (Ouahrani et al., 1993).

RNA composition Analysis of ribosomal RNA gene sequences has disclosed the unexpected phylogenetic position of the genus *Brucella* (De Ley et al., 1987; Dorsch et al., 1989). 5S rRNA gene sequence analysis indicates that *Brucella* is highly homogeneous, with about 95% relatedness to the *Agrobacterium* group but with a more distant relationship to *Bartonella*. The 16S rRNA gene sequence is typical of the *Alphaproteobacteria*. 16S rRNA/DNA binding has confirmed the close relationship to *Ochrobactrum* and

a rather more distant one to *Agrobacterium*, *Mycoplana*, and *Phyllobacterium*.

Extrachromosomal genetic elements Hitherto, there have been conflicting reports on the natural presence of plasmids or other forms of extrachromosomal DNA, such as transposons, in *Brucella*. Indirect evidence for the occurrence of plasmids, such as infectious antibiotic resistance, is also lacking. However, a small cryptic plasmid has been reported to be present in many strains. The existence of bacteriocins specific for the genus *Brucella* has not been confirmed, although bacteriocin-like effects have been occasionally reported (Pickett and Nelson, 1950; Todorov and Koleva-Todorova, 1971). Many bacteriophages active upon members of the genus *Brucella* have been described. These phages have not been shown to lyse bacteria of other genera and thus are of taxonomic value for identification at both genus and species level.

Phages and phage typing The phage-susceptibility pattern is of major importance in definition of the species of *Brucella*. For culture identification, it is convenient to use phage preparations standardized at the routine test dilution (RTD). This is defined as the minimum concentration that produces complete lysis of the propagating strain for that phage. It is essential that phage strains be maintained on the approved propagating strain, as the host range may be modified by adaptation to new strains.

Based on their host range, the phages may be classified into seven distinct groups. These are summarized in Table BXII.α.122.

The phages in group 1, typified by the Tbilisi (Tb) strain (Popkhadze and Abashidze, 1957), are capable of efficient replication only in cells of *B. abortus* in the S, SI, or I phases. Limited replication also occurs in S, SI, or I cultures of *B. neotomae*, but the efficiency of plating is low. At high concentrations, these phages produce lysis of S, SI, or I cultures of *B. suis* by a bacteriocin-like effect. Cultures of *B. melitensis*, *B. canis*, and *B. ovis* are not lysed, nor are cultures of *B. abortus*, *B. neotomae*, or *B. suis* in the M or R phases.

Group 2 comprises those phages typified by the Firenze (Fi) strain 75/13 (Corbel and Thomas, 1976). These replicate in, and form plaques on, S, SI, and I cultures of *B. abortus*, *B. neotomae*, and *B. suis* but not on those of other *Brucella* species. They are also inactive on M and R strains of *Brucella*. The efficiency of plating is somewhat higher on *B. abortus* strains than on those of *B. neotomae* or *B. suis* biovar 4, and very much higher on these than on *B. suis* biovars 1, 2, and 3.

Group 3 includes those phages typified by the Weybridge (Wb) strain (Morris and Corbel, 1973). All of these replicate and form plaques on S, SI, and I cultures of *B. abortus*, *B. neotomae*, and *B. suis*. The efficiency of plating on the three species varies between the phages of this group, within a range of about $2 \log_{10}$ units. Some of these phages have been reported to form plaques on cultures of *B. melitensis*, but the efficiency of plating is very low (Moreira-Jacob, 1968; Douglas and Elberg, 1976). None of the phages of group 3 will lyse M or R strains of any *Brucella* species, including *B. canis* and *B. ovis*.

Group 4 comprises the Berkeley phages Bk₀, Bk₁, and Bk₂ (Douglas and Elberg, 1976, 1978). The most useful of these, Bk₂, replicates and causes lysis in S-phase cultures of *B. abortus*, *B. melitensis*, *B. neotomae*, and *B. suis*. It shows no lytic activity towards *B. canis*, *B. ovis*, or other nonsmooth *Brucella* strains, even at high concentrations. Its lytic activity towards the smooth strains is drastically reduced as these undergo dissociation to nonsmooth forms. This is particularly the case with *B. melitensis* cultures, and

TABLE BXII.α.122. Lytic activity of phages for smooth (S) and rough (R) *Brucella* species^a

Phage Group	Phage strain	Titer	<i>B. melitensis</i>		<i>B. abortus</i>		<i>B. canis</i>	<i>B. neotomae</i>		<i>B. ovis</i>	<i>B. suis</i>	
			S	R	S	R	R	S	R	R	S	R
1	Tb	RTD	NL	NL	L	NL	NL	NL or PL	NL	NL	NL	NL
		RTD × 10 ⁴	NL	NL	L	NL	NL	L	NL	NL	L	NL
2	Fi 75/13	RTD	NL	NL	L	NL	NL	L	NL	NL	PL	NL
3	Wb	RTD	V	NL	L	NL	NL	L	NL	NL	L	NL
4	BK ₂	RTD	L or PL	NL	L	NL	NL	L	NL	NL	L	NL
5	R	RTD	NL	NL	V	L	NL	NL	NL	NL	NL	NL
	R/O	RTD	NL	NL	V	L	NL	V	NL	L	V	NL
	R/C	RTD	NL	NL	NL	L	L	NL	NL	L	NL	NL
6	Iz ₁	RTD	L or PL	V	L	NL	NL	L	PL	NL	L ^b	V ^b
											PL ^c	NL ^c
7	Np	RTD	NL	NL	L	NL	NL	NL	NL		NL	NL
		RTD × 10 ⁴	NL	NL	L	NL	NL	L	NL	NL	NL	NL

^aL, confluent lysis; PL, partial lysis, single plaques, or growth inhibition; NL, no lysis; V, variable, some strains lysed; RTD, routine test dilution.

^bBiogroups 1 and 4.

^cBiogroups 2, 3, and 5.

the efficiency of plating of Bk₂ phage on this species may vary considerably between strains.

Group 5 includes those phages lytic for nonsmooth *Brucella* strains. All are derived from phage R, which was developed as a mutant selected from a mixture of phages active on smooth *Brucella* strains (Corbel, 1977b, 1979). Phage R is lytic for nonsmooth cultures of *B. abortus* but not for other species. The strain is genetically unstable, however, and produces smooth-specific phage mutants at high frequency during replication. Phage R/O is lytic for *B. ovis* and some S-phase *B. abortus* and *B. suis* strains, but not for *B. melitensis* or nonsmooth cultures of *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, or *B. canis*. It is genetically unstable and its host range is subject to variation between successive batches. Phage R/M is lytic for some R-phase cultures of *B. melitensis* and, like phages R and R/O, is genetically unstable. Phage R/C was developed by passage of phage R/O on cultures of *B. canis* RM 6/66. Unlike the other phages in this group, it is relatively stable in host range and is therefore the most useful for taxonomic purposes. It is lytic for *B. canis*, *B. ovis*, and nonsmooth strains of *B. abortus*. Some nonsmooth strains of *B. suis* and *B. melitensis* are inhibited by it at high concentrations. It is not lytic for completely smooth strains of *B. abortus*, *B. melitensis*, *B. suis*, or *B. neotomae*, although it will produce plaques on strains of *B. abortus* in the SI, I, M, or R phases.

Group 6 is typified by the Izatnagar (Iz₁) phage. This is lytic for smooth cultures of all *Brucella* nomenclatures and produces patchy incomplete lysis on rough cultures of *B. melitensis*, *B. neotomae*, and *B. suis* (Corbel et al., 1988). The lytic activity towards nonsmooth cultures is rapidly lost if the phage is propagated on smooth cultures.

Group 7 is represented by the Nepean (Np) phages. These have a lytic spectrum similar to the Group 1 phages but produce complete lysis of *B. neotomae* at RTD (routine test dilution) and do not lyse *B. suis* at 10⁴ RTD (Rigby, 1990).

The brucellaphages are morphologically similar, with a hexagonal head 55–80 nm in width and a tubular, apparently non-contractile tail 14–33 nm long. Most of the phage strains have a mean head diameter of about 60 nm and a tail length of about 25 nm. Attachment of the phages is mediated via short fibrous structures linked to the distal end of the tail, which interact with protein or glycoprotein components forming part of the outer membrane of the bacteria (Corbel, 1977a). All of the brucellaphages are relatively stable to organic solvents and nonionic and

anionic detergents. They are inactivated by heat, cationic detergents, and oxidizing agents, show a variable stability to proteolytic enzymes and reducing agents, and do not require divalent cations for their interaction with *Brucella* cells. All contain DNA as their genetic material and, for the phages of group 1, this has a mol% G + C of 45.3–46.7 (*T_m*). In spite of their isolation from different sources and geographical areas over a wide range of time, their general properties and the results of restriction nuclease analysis indicate that the brucellaphages hitherto described probably comprise host range variants of a single phage (Rigby, 1990).

Although phage typing is used primarily for identification at the nomenclature level and usually gives a clear-cut result, some *Brucella* strains may show deviation from the standard pattern of lysis. This is particularly true for *B. melitensis* strains, which can be divided into subtypes according to pattern of susceptibility to Bk₂, Iz₁, and Wb phages (Corbel, 1987, 1989b).

Genetic exchange Chromosomal DNA exchange by conjugation has not been demonstrated between *Brucella* strains. This has impeded the application of classical methods of genetic analysis to the genus. However, transfer of plasmids by conjugation has been demonstrated between *Brucella* strains and *E. coli* under laboratory conditions. Tn5-associated kanamycin resistance has been transferred using a triparental cross protocol (Smith and Heffron, 1987). The IncP broad-host-range plasmid R751 encoding trimethoprim resistance has also been transferred from *E. coli* to reference strains of the six *Brucella* nomenclatures (Verger et al., 1993). Similar results were obtained with the broad-host-range plasmids pTH10 (IncP, Tc^R, Km^R), pSa (IncW, Tc^R, Km^R), and pR751 (IncP, Tp^R). The three plasmids were transferred successfully by conjugation from an *E. coli* donor to *B. abortus* S19, and were maintained in the transconjugant strain with no effects on biotyping properties. Moreover, plasmid transfer was also demonstrated from *B. abortus* to a recipient *E. coli* strain. In contrast, electroporation of narrow-range ColE1-derived plasmids failed to manifest a replicating plasmid, but rather facilitated transposon integration into the chromosome (Rigby and Fraser, 1989). Most recent molecular transformations of *B. abortus* have used the plasmid pBR1MCS (Kovach et al., 1994).

Variation Spontaneous variation in some of the properties of *Brucella* cultures is not uncommon. This usually results from mutations involving the modification of individual characteristics. Probably the most frequent mutation is the production of

nonsmooth variants from S-phase organisms. Inactivation of the perosamine synthetase gene results in production of stable rough mutants (Godfroid et al., 1998). Other characteristics are also subject to variation. CO₂-independent variants are rapidly selected from CO₂-dependent cultures of *B. abortus* and, much less frequently, from *B. ovis*. Loss of H₂S production and urease production occurs occasionally. Changes in resistance to various dyes, including basic fuchsin, thionine, thionine blue, safranin O, and to meso-erythritol, and various antibiotics, occur with varying frequency. Modification of such characteristics may result in alteration of biovars. For example, *B. abortus* biovar 2 can convert to biovar 1 by acquiring resistance to basic fuchsin; the mutation rate for resistance to this dye is 6.0×10^{-10} /cell division (Shibata et al., 1962). Variations in surface antigens or phage sensitivity in S-phase cultures occur infrequently; nevertheless, smooth phage-resistant variants of *B. abortus* have been isolated under both laboratory and field conditions (Corbel and Morris, 1974, 1975; Harrington et al., 1977).

In general, *Brucella* strains maintain their nomenclature identity, which is closely related to their preferred host specificity.

Cell wall-defective variants of *Brucella* may be induced by penicillin or glycine (Hines et al., 1964; Roux and Sassine, 1971; Hatten, 1973), hormones (Meyer, 1976), or cell cultures of immune macrophages (McGhee and Freeman, 1970a). They may also be recovered from the blood or tissues of animals or humans infected with *Brucella* strains (Nelson and Pickett, 1951; Ross and Corbel, 1980). Artificially induced spheroplasts are osmotically unstable and require hypertonic media for survival and growth. Naturally occurring *Brucella* L-forms are less osmotically sensitive but have more exacting growth requirements than the parent *Brucella* strains. Thus, they may require specially enriched media containing reducing agents (Nelson and Pickett, 1951) or high concentrations of horse serum (Corbel et al., 1980).

Cell wall-defective *Brucella* variants are highly pleomorphic and, if induced to grow on solid media, they produce bizarre colonies. These may vary from tenacious, granular, fried egg colonies of the *Mycoplasma* type, which may or may not be surrounded by a lipid film, to colonies resembling those of normal smooth or intermediate *Brucella* cultures, but very much smaller (Nelson and Pickett, 1951; Corbel et al., 1980). *Brucella* L-forms and spheroplasts normally show partial or complete loss of surface antigens but may also express surface antigens characteristic of nonsmooth cultures. They usually show a reduced sensitivity to brucellaphages but may be susceptible to growth inhibition or lysis. Their pathogenicity towards experimental animals is slight or absent, unless they revert to the parent form.

Antigenic structure Smooth species of *Brucella* show complete crossreaction in agglutination tests with unabsorbed antisera to smooth *Brucella* organisms. This crossreaction does not extend to nonsmooth variants in the M or R phases. Crossreactions between nonsmooth strains can be demonstrated by agglutination tests with unabsorbed antiserum to a rough *Brucella* strain or to the R antigen of *B. ovis*. By using crossabsorbed antisera, different quantitative distribution of two major surface epitopes, A and M, can be demonstrated in smooth strains (Wilson and Miles, 1932) and is of value in differentiating biovars of the major species (see Table BXII.α.120). It should be emphasized that this procedure should only be performed with high-quality monoclonal or polyclonal reagents of validated specificity. These antigenic determinants, together with the common C epi-

tope, are present on the O chain of the LPS, the immunodominant surface antigen of smooth *Brucella* species. The structure of this, and the related R LPS, is discussed under "Cellular composition".

An antigenic relationship, attributable to the presence of N-acylated-D-perosamine, has been confirmed between the LPS antigens of smooth *Brucella* species and *Yersinia enterocolitica* 0:9, *Francisella tularensis*, *Salmonella* 0:30 serotypes, *Vibrio cholerae*, and the 0:157 antigen of *E. coli* and *E. hermannii* (Corbel, 1985). The 8-kDa Braun lipoprotein also crossreacts with the homologous protein of *E. coli* (Gomez-Miguel et al., 1987).

Serological crossreactions between nonsmooth *Brucella* strains and organisms of other genera have received relatively little attention. Evidence for crossreactions between *B. canis* and *Actinobacillus equuli*, mucoid strains of *Pseudomonas aeruginosa*, and some serotypes of *Pasteurella multocida* has been presented (Weber, 1976; Carmichael et al., 1980). The outer membrane lipoproteins share antigenic determinants with *Agrobacterium* and other members of the former *Rhizobiaceae* (Cloeckaert et al., 1999).

Minor surface or subsurface antigens common to smooth and certain nonsmooth *Brucella* strains have been described. These include the "native hapten", also referred to as component 1 or second polysaccharide, present in phenol-water, ether-water, or trichloroacetic acid extracts of smooth strains and the rough strain *B. melitensis* B115 (Diaz et al., 1968; Moreno et al., 1981), which corresponds to free O chain (Cloeckaert et al., 1992). This is present in crude preparations of the polysaccharide B, which was formerly thought to be an antigen but is now known to be a nonantigenic cyclic beta-glucan (Perry and Bundle, 1990).

Many of the minor surface or subsurface antigens previously reported in smooth and some nonsmooth *Brucella* strains and designated as χ , β , and γ antigens, and the f antigen (Freeman et al., 1970; McGhee and Freeman, 1970b) have subsequently been identified with outer membrane proteins, bacterioferritin, or enzymes. Some of these proteins, including those with molecular masses of 10-, 16.5-, 19-, 25–27-, 36–38-, and 89-kDa, stimulate significant immune responses in infected individuals. Specific functions have been attached to some, such as the lumazine synthetase (Goldbaum et al., 1999), bacterioferritin (Denoel et al., 1995), and stress proteins (Robertson and Roop, 1999).

The soluble internal antigens released on disruption of *Brucella* cells can be demonstrated by immunodiffusion, immunoelectrophoresis, or immunoblotting. They are, in many cases, common to both smooth and nonsmooth strains. Some appear to be unique to the *Brucella* genus, but several at least are shared with other genera in the *Rhizobiales*, particularly *Ochrobactrum* (Velasco et al., 1998). Of particular importance is the L7/L12 ribosomal protein. This plays a key role in the induction of cell-mediated immunity and is a major protective antigen of *Brucella* (Bachrach et al., 1994a, b; Oliveira and Splitter, 1996).

Antibiotic sensitivity *Brucella* strains show sensitivity to a wide range of antimicrobial agents, but there is limited correlation between activity *in vitro* and therapeutic efficacy, mainly because the latter is influenced by pharmacodynamics, and sustained high intracellular levels are essential for eradication of the infection. Variations in susceptibility between nomenclature species, and even between biovars and strains within species, occur. Antibigram resistance patterns have been used for characterization at the strain level and have suggested a clonal structure, at least in *B. abortus* biovar 1 (Corbel, 1989a).

Sensitivity to beta-lactams is variable; some strains are sensitive to benzylpenicillin, ampicillin, and amoxicillin but most are resistant to methicillin, nafcillin, piperacillin, and ticarcillin. Similarly, sensitivity to cephalosporins tends to be limited except that for some third generation agents such as cefotaxime, ceftizoxime, and ceftriaxone, MICs are in the range 0.25–2 mg/l. Sensitivity to most macrolides is low but clarithromycin and azithromycin are active in the range 2–8 mg/l. The MIC for chloramphenicol is 2–3 mg/l for most strains. For rifampicin and rifapentine, MICs are in the range 0.1–2 mg/l. Similarly, sensitivity to tetracyclines is generally about 0.1 mg/l.

Nearly all strains are resistant to nalidixic acid, but MICs for fluoroquinolones are in the range 0.5–1 mg/l. Sensitivity to cotrimoxazole is borderline. Most *Brucella* strains are resistant to polymyxins, amphotericin B, bacitracin, cycloheximide, clindamycin, lincomycin, nystatin, and vancomycin at concentrations that inhibit many other organisms, and this fact has allowed the formulation of selective culture media (Farrell, 1974). *Brucella* strains are sensitive to the rhizobial peptide trifolitoxin, and on this basis have been classified in the bacteria known as Group 1 of the alpha-2 *Proteobacteria* (Triplett et al., 1994).

Pathogenicity *Brucella* species are pathogenic for a wide variety of animals, frequently producing generalized infections with a bacteremic phase followed by localization in the reproductive organs and the reticuloendothelial system. Infection in the pregnant animal often results in placental and fetal infection and this frequently causes abortion. The organisms may localize in mammary tissue and can be excreted in the milk. Because all the main species of meat- and milk-producing domesticated animals are susceptible to brucellosis and act as sources of human infection, the economic impact of the disease is enormous.

Typically, growth *in vivo* is intracellular and the organisms can survive within both granulocytes and monocytes. Infections in the natural host are rarely lethal and often mild, with clinical manifestations occurring mainly in the pregnant animal. Nevertheless, localization can occur in a wide range of organs with production of a variety of lesions.

All the *Brucella* species may produce infection in laboratory animals including guinea pigs, mice, and rabbits, but the severity of the infection varies considerably with the virulence of the infecting strain. For the guinea pig, the order of pathogenicity is *B. melitensis* > *B. suis* > *B. abortus* > *B. neotomae* > *B. canis* > *B. ovis* (Braude, 1951; Isayama et al., 1977).

The more pathogenic strains usually produce a local abscess at the site of inoculation, followed by bacteremia of varying duration. The regional lymph nodes may become enlarged and granulomatous changes develop. Similar changes occur in the liver and spleen and frequently in other organs, particularly the testes and epididymides. *B. melitensis* and *B. suis* biovars sometimes produce fatal infections (Braude, 1951). The other species rarely produce severe disease, and infection is usually self-limiting within a period varying from a few weeks to more than 6 months. The mouse is more susceptible to persistent infection with *B. neotomae*, *B. canis*, and *B. ovis* than the guinea pig (Isayama et al., 1977). Pathogenic effects of these nomenclatures are limited to slight-to-moderate splenic enlargement.

Infection in humans arises from direct or indirect contact with infected animals or by consumption of milk or meat products derived from them. Person-to-person transmission is extremely rare and plays no part in the natural history of the disease. Entry

of the organisms may be via the respiratory or gastrointestinal mucosa, or the percutaneous route. The subsequent pathogenesis is believed to follow a similar pattern to that observed in experimental animals, with proliferation in lymphoid tissue succeeded by a bacteremic phase of variable duration with, in some cases, localization in specific organs. The infection may be completely subclinical, or it can produce a subacute or acute febrile illness. In the absence of adequate antibiotic therapy, this can persist for many months and may be accompanied by the development of severe complications such as endocarditis, meningoencephalitis, arthritis, spondylitis, and orchitis. A postinfectious, chronic, debilitating syndrome may also result. *B. melitensis* accounts for the majority of severe infections, followed by *B. suis*. *B. abortus* and *B. canis* are usually associated with milder disease. Infection elicits both antibodies and cell-mediated immunity. The LPS is the dominant antigen in the serological response but antibodies and delayed hypersensitivity to a variety of proteins can develop. Further details are given by Young and Corbel (1989) and Madkour (2000).

Ecology Although easily cultivated *in vitro*, under natural conditions *Brucella* species behave as obligate parasites and do not pursue an existence independent of their animal hosts. However, under suitable conditions of temperature and humidity they can persist in the environment, for example in soil or surface water, for long periods. Their distribution is worldwide, apart from the few countries from which they have been successfully eradicated. Considerable local variations in the prevalence of particular nomenclatures and biovars occur. This is of epidemiological importance (Corbel, 1989a).

ENRICHMENT AND ISOLATION PROCEDURES

The isolation of *Brucella* may be attempted from any tissue or secretion. Those most likely to yield positive cultures include abortion material (placental cotyledon, amniotic fluid, vaginal discharge, fetal gastric contents, fetal lung, and fetal liver), lymph nodes, bone marrow, mammary gland, uterus, seminal vesicles and accessory glands, testes, and epididymides, or other organs with local lesions, milk, colostrum, semen, and blood.

Uncontaminated materials may be inoculated directly on to SDA plates. Where contaminating organisms are likely to be present, a selective medium should be used. SDA supplemented with the antibiotic formulation of Farrell (1974)⁶ is satisfactory for the isolation of most smooth strains of *Brucella*, but may be too inhibitory for *B. canis* and *B. ovis*. For the isolation of these species from contaminated material, modified Thayer–Martin medium (Brown et al., 1971), or SDA containing 10% (v/v) heated horse serum and VCN-F inhibitor (BBL),⁷ may be used. These media are less selective than Farrell's SDA, and it is advisable to dilute heavily contaminated material, such as semen, in 5–10 volumes

6. Farrell's selective medium: prepared from SDA. After addition of horse serum and glucose to the molten medium cooled to 56°C, antibiotics are added to give the following final concentrations: bacitracin, 25 U/ml; polymyxin B, 5 U/ml; cyclohexamide, 100 µg/ml; vancomycin, 20 µg/ml; nalidixic acid, 5 µg/ml; and nystatin, 100 U/ml.

7. Modified SDA antibiotic medium: SDA is prepared as previously described, but with horse serum added to a final concentration of 10% (v/v) and No. 1 agar (Oxoid) added to give a final concentration of 3% (w/v). To each liter of molten medium cooled to 56°C, one vial of VCN inhibitor (Difco; reconstituted in 10 ml of sterile distilled water) is added. This is followed by addition of 1 ml furadantin solution (10 mg/ml of 0.1 N NaOH), and the plates are poured immediately. The medium contains vancomycin (3 µg/ml), sodium colistimethate (7.5 µg/ml), nystatin (12.5 U/ml), and furadantin (10 µg/ml) (final concentrations).

of sterile isotonic solution and filter it through a membrane filter (0.8 μ m pore size) before plating it out. All cultures should be incubated at 37°C under air supplemented with 5–10% (v/v) CO₂—unless isolation of a CO₂-independent species of *Brucella* is being attempted.

Direct inoculation of selective media is usually satisfactory for isolation of *Brucella* from heavily infected materials, even in the presence of contaminating organisms. However, when the *Brucella* concentration is likely to be low, as in blood, milk, or semen samples, enrichment procedures should be used. Incubation of milk samples at 4°C concentrates *Brucella* cells in the cream layer, which should then be cultured.

Enrichment may be achieved either by intramuscular inoculation of the samples into guinea pigs, followed by culture of the spleen tissue four weeks later, or by growth in a liquid enrichment medium. Guinea pig inoculation is only likely to succeed with virulent strains of *B. melitensis*, *B. abortus*, and *B. suis*.

Enrichment cultures may be performed by the two-phase system of Castañeda (1947). As liquid phase, SDB supplemented with 25% (w/v) trisodium citrate is used. The solid phase is SDA containing 2.5% (w/v) agar. The medium is made selective by adding the antibiotic formulation of Farrell (1974) to the liquid phase.

For cultures of *B. ovis* and *B. canis* the antibiotic formulation may be replaced by VCN-F inhibitor. The sample is mixed with the liquid phase and the bottles incubated at 37°C under air (with supplementary CO₂ if required). At intervals of 2–3 days, the liquid phase is tipped over the solid phase and the bottles re-incubated. Incubation is continued for up to 6 weeks or until colonies appear on the solid phase, whichever is sooner.

Blood or tissue samples may be enriched for *B. canis* by inoculation of the yolk sacs of 6–8-day-old chick embryos. Yolk from eggs with dead embryos is plated onto SDA or other suitable medium. Culture of cell-wall-defective forms of *Brucella* may be attempted by culturing samples of blood, synovial fluid, and solid tissue on Farrell's selective medium enriched with 20% (v/v) horse serum. Incubation should be continued for at least 14 days before discarding the plates. The plates should be examined for microcolonies under a stereomicroscope.

MAINTENANCE PROCEDURES

Cultures may be maintained for short periods by streaking onto SDA slopes, incubating for 72 h under air (+ 10% CO₂, v/v, if required) and then sealing the slope and storing at 4°C. This procedure needs to be repeated every 6–8 weeks. It is unsatisfactory for long-term maintenance of strains, as these are liable to change their characteristics on repeated subculturing.

Brucella cultures may be preserved satisfactorily by vacuum drying. The strains are grown on SDA slopes incubated at 37°C under air (+ 10% CO₂, if required) for 72 h. The growth is washed off the slopes and suspended in sterile rabbit serum to give a suspension of about 10¹⁰ organisms/ml. This is distributed in 0.1-ml volumes into sterile tubes sealed with cotton-wool plugs. The tubes are stored over phosphorus pentoxide in a desiccator kept at 4°C. The desiccator is evacuated daily until a pressure of 0.05-mm Hg can be obtained. The tubes are then sealed under vacuum in glass ampoules containing a small quantity of silica gel. Under these conditions, *Brucella* cultures may remain viable for many years.

Brucella strains may also be preserved satisfactorily by freeze-drying. General directions for this have been given by Lapage

et al. (1970) and details of the technique by Boyce and Edgar (1966).

Storage in liquid nitrogen will maintain *Brucella* cultures with a smaller decrease in viability than is produced by either vacuum drying or freeze-drying (Davies et al., 1973). Cultures are grown for 72 h on SDA slopes at 37°C in air (+ 10% CO₂, if required) and the growth suspended in single-strength Bacto-glutamate medium⁸ to form a dense suspension. This is left undisturbed at 4°C for 7 days, after which volumes of up to 1 ml are placed in sterile glass screw-capped vials. The vials are allowed to equilibrate in the vapor phase or immersed in the liquid.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Safety precautions All *Brucella* isolates should be regarded as potentially pathogenic for man. Adequate precautions against accidental infection should be taken at all stages of work involving live cultures. It is strongly recommended that all such procedures be performed by suitably trained staff using efficient exhaust protective cabinets. Particularly hazardous techniques, such as the determination of oxidative metabolism rates by respirometric methods, are best left to reference laboratories experienced in their use. Where possible, hazardous procedures should be replaced by methods that do not require viable bacteria or else are easily contained. Specific indications of safety precautions to be adopted during work with *Brucella* cultures have been given by Corbel et al. (1979) and Alton et al., (1988).

Oxidative metabolism rates For respirometric methods, cells are grown in Roux flasks containing SDA for 48 h. They are harvested in Sorensen's phosphate buffer (pH 7.0), washed 3 times by centrifugation (10,000 \times g for 15 min), and standardized turbidimetrically to a cell concentration equivalent to 0.9 mg N/ml, as determined by micro-Kjeldahl analysis. Measurements of oxygen uptake are performed using either a Warburg constant volume respirometer or a Gilson differential respirometer (see *Manometric Techniques* by Umbreit et al., 1972). Oxygen uptake with various substrates is expressed as μ l/mg of cell nitrogen per hour (Q_{O₂}N). The following substrates are normally used for *Brucella* studies: group 1: L-alanine, L-asparagine, and L-glutamic acid; group 2: L-arginine, DL-citrulline, DL-ornithine, and L-lysine; group 3: L-arabinose, D-galactose, D-glucose, D-ribose, D-xylose, and meso-erythritol. Other substrates are sometimes used. The substrates are prepared as 10% (w/v) solutions in Sorensen's phosphate buffer (pH 7.0), sterilized by filtration, and stored at –20°C until needed. The DL-citrulline solution should be sterilized by autoclaving at 121°C for 15 min.

The Q_{O₂}N values obtained by either the Gilson or the Warburg method are corrected for endogenous O₂ uptake (by subtracting the Q_{O₂}N value for controls lacking substrate) and are compared with expected values for each species (Meyer and Cameron, 1961a, b; Meyer, 1969; Verger and Grayon, 1977). The results may be conveniently expressed in the form of a three-level metabolic profile (Verger and Grayon, 1977) or as a pattern of + and – signs (see Table BXII.α.121).

Thin-layer chromatography The oxidative metabolic pattern may be determined semi-quantitatively by thin-layer chromatog-

8. Bacto-glutamate medium: Bacto-casitone (Difco) or equivalent, 25 g, is dissolved in 100 ml of distilled water and autoclaved at 115°C for 20 min. To this solution, 50 g of sucrose and 10 g of monosodium glutamate are added and dissolved by steaming for 10 min. The medium is filtered under positive pressure through Seitz EKS filters into sterile containers and finally autoclaved at 106°C for 15 min.

raphy. The results do not always coincide precisely with those obtained by respirometry, possibly because nonoxidative degradation of substrates may occur; however, the overall pattern is generally satisfactory for culture identification. The method used is a modification of that described by Balke et al. (1977). The amino acid and carbohydrate substrates are incubated with the *Brucella* suspension, and the supernatant fluids are then tested for the presence of the substrate. It is essential to include *Brucella* reference strains as standards during such testing. Details of the technique are given by Corbel and Brinley-Morgan (1984).

Substrate-specific tetrazolium reduction The method of Broughton and Jahans (1997) can be performed under containment conditions and is much more convenient and safer than respirometry. The strains are cultured on Trypticase soy agar (or equivalent) layers for 48–72 h at 37°C in 10% CO₂. The bacteria are then harvested in phosphate-buffered saline, pH 7.2 and adjusted turbidimetrically to a concentration equivalent to 10¹⁰ organisms/ml. The substrates L-alanine, L-asparagine, L-glutamic acid, L-arginine, DL-ornithine, L-lysine, D-galactose, D-ribose, D-xylose, *meso*-erythritol, and urocanic acid are prepared as separate solutions in PBS at 20 g/l, adjusted to pH 7.2 if required, and sterilized by membrane filtration. These stock solutions are stored at 4°C and diluted to 1.25 g/l in sterile PBS immediately before use. Each column in a flat-bottomed microtiter plate is loaded with 100 µl of substrate. Volumes of 50 µl of each cell suspension are added to four rows of wells, and the plates incubated at 37°C in 10% CO₂ for 18 h. After incubation, 50 µl of a sterile solution of 1 g/l of 3-[4,5-dimethylthiazol-2yl] 2,5 tetrazolium bromide (MTT) in PBS is added to each well. After 1 h at room temperature (18–22°C), 50 µl of 40% formaldehyde is added to each well and after a further 2–4 h, the optical density of each well is determined using a microplate reader at 630 nm. The values for the four replicates of each substrate are calculated. Comparisons are performed with reference strains included in each run. The pattern of utilization of substrates is similar to that obtained with respirometry, but the oxidation of urocanate distinguishes *B. suis* from *B. canis*.

Determination of phage sensitivity The colonial phase of the test culture must first be determined by streaking GDA plates and incubating under a suitable gas atmosphere at 37°C for up to 5 days. The colonial morphology is then examined in obliquely transmitted light according to Henry (1933) or after ammonium oxalate-crystal violet staining according to White and Wilson (1951). If the culture has colonies of only a single phase, representatives of these are selected for phage typing. If a mixture of smooth and nonsmooth colonial forms is present, then smooth colonies are selected for subculturing and phage typing.

For routine phage typing, a suspension of cells is prepared as described for the slide agglutination test (see below). Plates of SDA or TSA are inoculated with this suspension so as to produce confluent growth. Typing may be performed using the Tb phage standardized at the routine test dilution (RTD) and at 10,000 × RTD, or by using phages representing groups 1–5 of Corbel and Thomas (1980) standardized at RTD. For typing smooth cultures, Tb phage alone, when used at both concentrations, is usually adequate, but for nonsmooth or atypical cultures, the additional phages are useful. Discrete drops of phage preparation (~25 µl) are applied to the surface of the inoculated plate and allowed to soak in the agar. The plates are then inverted and incubated for 37°C under the appropriate gas atmosphere. They are in-

spected for growth and phage lysis after 24 h incubation, and at further 24 h intervals if required. The results of the phage sensitivity test can only be interpreted accurately if the colonial phase of the culture is known. In addition to the initial examination of the colonial morphology of the culture, a further check should be done by testing a sample of growth from each phage plate for agglutinability in 0.1% aqueous acriflavine (clumping indicates nonsmooth cells).

CO₂ requirement The culture is streaked onto duplicate SDA slopes. One of these is incubated at 37°C under an air atmosphere, the other under air supplemented with 5–10% CO₂. Growth on both slopes indicates an absence of CO₂-dependence, whereas growth only with CO₂ supplementation indicates a CO₂ requirement. The test should be performed on freshly isolated cultures as CO₂-dependence can be rapidly lost by *Brucella* strains.

H₂S production The culture is inoculated onto an SDA or TSA slope, and a strip of lead acetate-impregnated paper is placed in the mouth of the tube. This must not come into contact with the medium or with condensation on the wall of the tube. The culture is incubated under the appropriate atmosphere for 4–5 days. The paper strip is examined and changed daily. Blackening of the paper indicates H₂S production. Slight blackening of the tip of the paper for the first day only is not considered positive.

Agglutination with monospecific antisera The growth from an SDA slope incubated for 48 h is suspended in ~0.5 ml of sterile saline to give an opacity equivalent to ~10¹⁰ cells/ml. One drop of this suspension is mixed with an equal volume of 0.1% aqueous acriflavine on a glass slide and examined for agglutination. The absence of agglutination indicates a smooth culture. In this case, one drop of the suspension is added to each of a series of drops of A and M monospecific antisera, and to negative control serum, on a glass slide. After agitation for 1 min, each drop is examined for agglutination. Smooth *Brucella* cultures will produce agglutination with either A and/or M sera, but not with the negative control serum.

If the culture is nonsmooth (agglutination occurs with acriflavine), one drop of culture suspension is added to a drop of R antiserum and to a drop of negative control serum, with subsequent agitation for 1 min. The absence of agglutination in either serum will exclude a nonsmooth *Brucella* strain. Agglutination with only the R antiserum is strongly suggestive of a nonsmooth *Brucella*. Agglutination with both sera may also indicate nonsmooth *Brucella*, although rough organisms of other genera occasionally react in this way.

Urease activity Christensen's agar slopes are each inoculated with a single loopful of culture suspension prepared as described for the slide agglutination tests. The slopes are examined immediately and after 15 min, 1 h, 2 h, and 24 h incubation at 37°C. Cultures of *B. suis*, *B. canis*, and *B. neotomae* almost invariably produce an immediate positive reaction, turning the medium magenta within 15 min. Most strains of *B. abortus* and *B. melitensis* will give positive reactions after 1–2 h and nearly all after 18–24 h. The reference strain *B. abortus* 544 is an exception and is urease-negative even at 24 h. *B. ovis* cultures are also urease-negative under these conditions, although some strains will give positive reactions if incubated for up to 7 days.

Growth in the presence of dyes Cell suspensions prepared as for the slide agglutination tests are used to inoculate plates of SDA or other basal media containing the dyes basic fuchsin

or thionine⁹. The plates are divided into four quadrants and one loopful of suspension is applied to each quadrant and streaked five times in succession without recharging the loop. The plates are incubated at 37°C under the appropriate gas atmosphere for up to four days. Growth on three or more streaks is considered to indicate resistance to the dye. Growth on only one or two streaks is not considered significant. Other dyes, such as safranin, methyl violet, pyronine Y, thionine blue, or malachite green, are occasionally used in addition to basic fuchsin and thionine. In each case, it is essential that plates inoculated with the biovar 1 reference strains for each *Brucella* species are incubated and examined in parallel with the test cultures.

Nucleic acid amplification procedures Procedures using the polymerase chain reaction (PCR) are being used increasingly for diagnostic purposes. Because of their speed, sensitivity, and safety they are likely to replace culturing methods in many instances. This means that in the future, fewer strains are likely to be available for conventional typing. A useful degree of typing is achievable by molecular methods if attention is paid to the selection of suitable primers. Use of the rRNA gene spacer region (*rrs-rrl*) and IS6501 allows identification at genus level. Selection of primers for the *omp2a*, *2b*, and *25* genes will permit putative identification to nomenclature and, in many cases, biovar level. The procedures used are those described in the original reports (Halling and Zehr, 1990; Cloeckaert et al., 1995; Ficht et al., 1996; Fox et al., 1998c).

DIFFERENTIATION OF THE GENUS *BRUCELLA* FROM OTHER GENERA

Most of the bacterial species known to cross-react serologically with *Brucella* are unlikely to be misidentified as members of this genus, as they are easily distinguishable by their morphological, cultural, and biochemical characteristics. A possible exception is *F. tularensis*; however, this organism is unlikely to grow on ordinary brucella culture media, is not susceptible to any known brucella phages, and is usually very much smaller than *Brucella* cells. It also shows fermentative activity towards glucose and a variety of other carbohydrates, is catalase negative, has a low cytochrome *c* content, produces rapidly lethal infections in mice, and does not share internal antigens with *Brucella* strains (although unabsorbed antisera will cross-react in agglutination and immunofluorescence tests). Moreover, its DNA base composition (33–36 mol% G + C) is quite distinct from that of *Brucella*, and there is no relatedness of polynucleotide sequences via DNA–DNA hybridization (Hoyer and McCullough, 1968b).

Confusion is most likely to occur with other small, Gram-negative, nonfermentative bacteria. These include *Bordetella* species, particularly *Bordetella bronchiseptica*, which is often isolated from animal sources, and species of *Achromobacter*, *Acinetobacter*, *Branhamella*, *Kingella*, *Moraxella*, and *Neisseria*. Some of the less frequently identified *Pseudomonas* species have also been con-

fused with *Brucella*, as have occasional strains of *Haemophilus* and *Pasteurella*.

Differentiation of these taxa is achieved in the first instance by careful examination of Gram-stained smears. The morphology of many of these organisms is usually sufficiently distinctive to differentiate them from *Brucella*. Examination of growth and colonial morphology on brucella culture media incubated at 20°C and 37°C, and motility tests at both these temperatures, will enable many other isolates to be eliminated.

At this stage the colonial phase of a suspected *Brucella* strain should be determined. Slide agglutination tests with unabsorbed antiserum to smooth and nonsmooth *Brucella* strains will then permit recognition of true members of the genus in nearly every case. Occasionally, nonsmooth organisms other than *Brucella* are agglutinated by antisera to nonsmooth *Brucella* strains. Subsequent tests with phage R/C will permit *B. canis*, *B. ovis*, and nonsmooth *B. abortus* strains to be definitely identified as members of the genus. The identification of nonsmooth strains of the other species can occasionally present difficulties, particularly if they give atypical results in one or more of the routine typing procedures. In such cases it is necessary to resort to further tests for genus identification. These may include pulsed-field gel electrophoresis of DNA extracts (Allardet-Servent et al., 1988), PCR using genus-specific primers (Halling and Zehr, 1990; Cloeckaert et al., 1995; Fox et al. 1998c), gas-liquid chromatography of the fatty acid methyl esters (Tanaka et al., 1977), and serological demonstration of genus-specific antigens by immunodiffusion or immunoblotting (Velasco et al., 1998).

TAXONOMIC COMMENTS

Molecular genetic studies, particularly those involving sequencing and hybridization of the ribosomal RNA fractions, have clearly demonstrated that the genus *Brucella* forms a discrete homogeneous group, which, of known taxa, is most closely related to the genus *Ochrobactrum*. It quite clearly belongs in the *Alphaproteobacteria*. Sequencing of 5S and 16S rRNA and competitive binding studies of these molecules with DNA (De Ley et al., 1987; Dorsch et al., 1989; Relman et al., 1992), lipid composition (Weckesser and Mayer, 1988), antigenic relatedness (Velasco et al., 1998; Cloeckaert et al., 1999), and multi-locus enzyme electrophoresis have confirmed a close genetic relationship to *Ochrobactrum* and, to a lesser extent, *Agrobacterium*, *Mycoplana*, and *Phyllobacterium*, and a somewhat more distant relationship to *Bartonella*. *Brucella* also shares gene sequences essential for pathogenicity with *Agrobacterium* (Sola-Landa et al., 1998; O'Callaghan et al., 1999). The cyclic glucan synthetase gene is also shared with other species within the *Rhizobiales* (De Iannino et al., 1998). The phylogenetic relationships summarized by Yanagi and Yamasato (1993) suggested an affiliation to the *Rhizobiaceae*. However, a more recent analysis of this family indicates that *Brucella* and *Ochrobactrum* form a discrete group outside it (Fig. BXII.α.138). This suggests that until evidence to the contrary is presented, these two genera and *Mycoplana* should represent the only known members of the *Brucellaceae*.

The issue of subdivision within the genus is less clear-cut. DNA–DNA binding studies within the genus have shown all nomenclature to share similar polynucleotide sequences; competitive binding assays have indicated a relatedness of >90% (Hoyer and McCullough, 1968a, b; Verger et al., 1985). This degree of DNA relatedness is indicative of a very close genetic similarity between all members of the genus and is consistent with the concept of a single species. This conclusion has been supported

9. Dye sensitivity test media: a 0.1% solution of basic fuchsin or thionine is prepared in distilled water and heated at 100°C for 1 h. Portions of this stock solution are then added to molten basal media such as SDA or TSA so as to produce a final dye concentration between 10 and 40 µg/ml. The exact concentration required to produce satisfactory differentiation of biovars must be determined for each batch of dye medium by use of *Brucella* reference strains. The plates should be incubated overnight at 37°C before use, to reveal any contaminants. Dyes from National Aniline Division, Allied Chemical and Dye Co., New York, NY, USA were originally recommended but are no longer available. Dyes from other sources may be used but each batch must be validated before use. Basic fuchsin is now difficult to obtain and has been replaced by *p*-rosaniline.

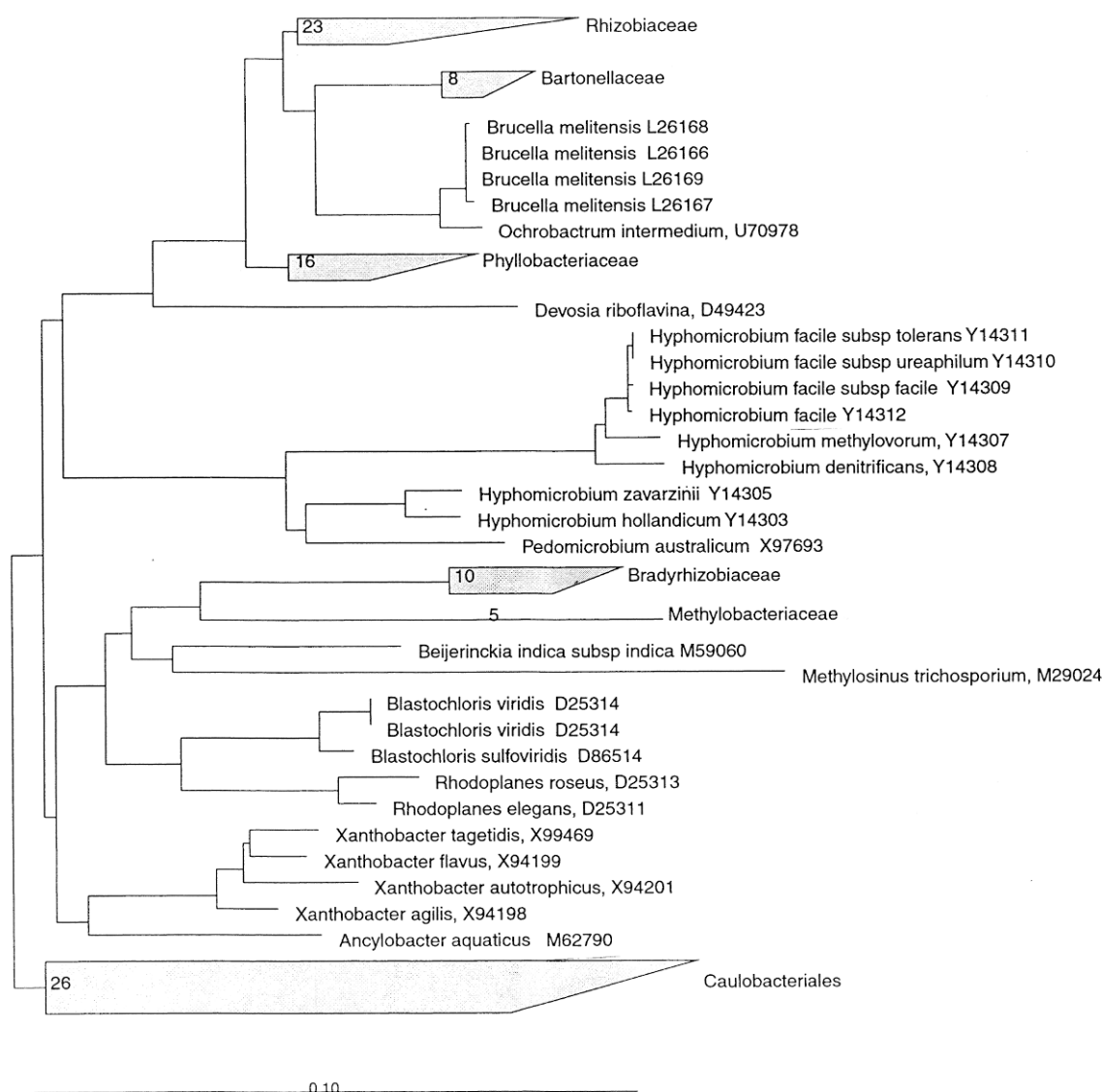


FIGURE BXII.α.138. Phylogenetic tree showing the relationships between the families of *Rhizobiaceae*, *Bartonellaceae*, *Bradyrhizobiaceae*, *Methylobacteriaceae*, and the order *Caulobacteriales*, prepared using the weighted least squares method implemented by PAUP, with inverse-squared weighting. Distances were calculated using 1182 positions and corrected according to the HKY85 model (Courtesy of the Ribosomal Database Project).

by gene sequencing and genome mapping as indicated above. These have shown that the various nomenclatures share an essentially similar genomic structure, the main differences being in arrangement rather than content. Current evidence suggests that the presently recognizable subdivisions evolved from a precursor monochromosomal strain resembling *B. suis* biovar 3. The double chromosomes present in the other nomenclatures and biovars contain very similar genes to those represented in the single chromosome, suggesting that they arose through scission of the latter. This emphasizes the essential unity of the genus. Nevertheless significant differences in the genetic, biochemical, cultural, and pathogenic properties of the strains corresponding to its recognized subdivisions do exist, and these are correlated with host specificity (Fig. BXII.α.139). While the concept of separate species is not tenable, it is clear that subdivision at a level higher than biovar should be maintained. To minimize the confusion that would be likely to result from the adoption of a completely novel nomenclature, it is proposed that the current di-

vision into nomenclatures should be retained until an adequate genetically based classification is devised.

The present system of classification was devised by the Subcommittee on Taxonomy of the genus *Brucella* at its first meeting (Stableforth and Jones, 1963), with amendments introduced at its subsequent meetings (Jones, 1967; Jones and Wundt, 1971; Wundt and Morgan, 1975). Although a number of studies have produced suggestions for modifications of this scheme, none of the proposed changes has yet emerged as entirely satisfactory. Any improved system needs to take into account the genetic evidence indicating the monospecific nature of the genus, but at the same time acknowledge the significant divisions which exist below species level, particularly in relation to genomic arrangements and host specificity. At this level, the main problem is one of terminology, as clearly differences are discernible within the genus at a higher level than that of the conventionally defined biovars. The application of genetic typing methods has not resolved this problem; rather it has added a further dimension of

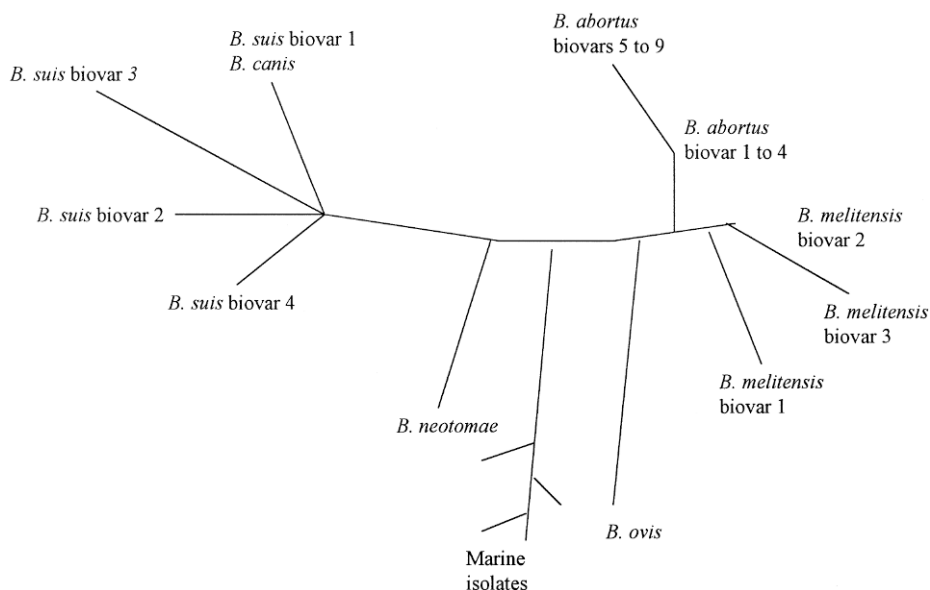


FIGURE BXII.α.139. Phylogenetic tree of the *Brucella* species.

complexity as the level of subdivision can be increased progressively by increasing the number of gene polymorphisms examined.

The biovars defined by conventional biotyping tests correspond to epidemiologically significant variants and this scheme is still useful, although it is evidently incomplete. It is also internally inconsistent, as the number of differential characteristics necessary to define a biovar is variable. For example, the conventional scheme identifies only three biovars of *B. melitensis* based on serology. In fact, strains of this organism may show a similar range of sensitivity to basic fuchsin and thionine to that shown by *B. abortus* strains and this extends the number of possible biovars to twelve, all of which have been detected at some time (Banai et al., 1990; Corbel, 1991; Bardenstein et al., 2002). Similarly, strains of the other nomenspecies that do not fit into the current biotyping scheme have also been described but not accorded separate status.

The conventional scheme also needs to be extended to ac-

commodate the strains isolated from marine mammals. These differ from the currently recognized nomenspecies by as much as the latter differ from each other. It is also clear that distinct groups exist within the marine strains, corresponding to isolations from seals, porpoises, dolphins, or whales (Ross et al., 1996; Clavareau et al., 1998; Miller et al., 1999). These distinctive types may need to be regarded as separate nomenspecies. They show some divergence from the “mainstream” nomenspecies and quite clearly form a distinct branch of the genus (Fig. BXII.α.139).

At one point, the designation “*B. maris*” was suggested for the marine isolates. However, both conventional and genotyping methods indicate the heterogeneity of the group. The marine strains break down into four clear subgroups corresponding to oxidative metabolism pattern and preferred host species. These are distributed across seven marine-specific genotypes according to *omp2* and *omp25* PCR-RFLP typing. The official status of these isolates has yet to be decided but they undoubtedly comprise a novel subdivision within the genus *Brucella*.

List of species of the genus *Brucella*

1. ***Brucella melitensis*** (Hughes 1893) Meyer and Shaw 1920, 179^{AL} (*Streptococcus melitensis* Hughes 1893, 235).
me.li.ten'sis. L. adj. *Melitensis* of or pertaining to the Island of Malta (Melita) where first isolated (Bruce, 1893).

The morphological and cultural characteristics are as described for the genus. Other characteristics of the species and its biovars are indicated in Tables BXII.α.120 and BXII.α.121. The usual natural hosts are sheep and goats, but other species, including cattle, pigs, and humans, may be infected. Smooth cultures are usually pathogenic for the guinea pig and the mouse. In general, smooth cultures of *B. melitensis* tend to be more virulent for laboratory animals than *B. abortus* and may cause fatal infections. Nonsmooth cultures are usually avirulent for both laboratory animals and the natural hosts.

The mol% G + C of the DNA is: 58 (T_m).

Type strain: 16M, ATCC 23456, NCTC 10094.

GenBank accession number (16S rRNA): L26166.

Additional Remarks: The complete genome of strain 16M has been published by DeVecchio et al. (2002). Strain 16M is also the reference strain for biovar 1. The reference strain for biovar 2 is 63/9 (ATCC 23457; NCTC 10508), and that for biovar 3 is Ether (ATCC 23458; NCTC 10509).

2. ***Brucella abortus*** (Schmidt 1901) Meyer and Shaw 1920, 176^{AL} (*Bacterium abortus* Schmidt in Schmidt and Weiss 1901, 266).
a.bor'tus. L. gen. n. *abortus* of abortion, miscarriage; first isolated by Bang (1897).

The morphological and cultural characteristics are as described for the genus. Other characteristics of the species and its biovars are indicated in Tables BXII.α.120 and BXII.α.121. The usual natural hosts are cattle and other bovidae. Horses, camels, sheep, deer, dogs, man, and other species may also be infected. Placentitis and abortion are usually produced in the pregnant animal. Pathogenic for

laboratory animals including rabbits, guinea pigs, and mice; the guinea pig is probably the most susceptible. Rough strains are usually avirulent, but mice retain the organisms in the spleen for some time after inoculation.

The mol% G + C of the DNA is: 56 (Bd) or 57 (T_m).

Type strain: 544, ATCC 23448, NCTC 10093.

Additional Remarks: ATCC 23448 is also the reference strain for biovar 1. For biovar 2 (referred to as "dye-sensitive *B. abortus*" by Wilson, 1933) the reference strain is 86/8/59 (ATCC 23449; NCTC 10501). For biovar 3 (referred to as "Rhodesian *abortus*", "non-CO₂-requiring, thionine-resistant *B. abortus*" (Bevan, 1930) or "CO₂-requiring, thionine-resistant *B. abortus*" (Van der Schaaf and Rosa, 1940), the reference strain is Tulya (ATCC 23450; NCTC 10502). The reference strain for biovar 4 is 292 (ATCC 23451; NCTC 10503). For biovar 5 (referred to as British *melitensis* [Stableforth, 1959]) the reference strain is B3196 (ATCC 23452; NCTC 10504). The reference strain for biovar 6 is 870 (ATCC 23453; NCTC 10505). The reference strain for biovar 7, 63/75 (ATCC 23454; NCTC 10506), was reported to be a mixture of both A- and M-dominant strains and this biovar has been suspended. For biovar 9 (referred to as "H₂S-producing *B. melitensis*" by Taylor et al., 1932) the reference strain is C68 (ATCC 23455; NCTC 10507). Biovar 8 was originally described as "CO₂-requiring *B. melitensis*" by Taylor et al. (1932), but as no cultures of this biovar are known to exist, the status of this biovar was suspended by the Subcommittee on the Taxonomy of the genus *Brucella* in 1978 (Corbel, 1982).

3. ***Brucella canis*** Carmichael and Bruner 1968, 579^{AL}
ca'nis. L. gen. n. *canis* of the dog.

The morphological and cultural characteristics are as described for the genus. Other characteristics are indicated in Tables BXII.α.120 and BXII.α.121. No biovars are recognized. On incubation for more than a few days, the colonies become very tenacious and viscous. The growth is almost impossible to emulsify and forms a ropy agglutinate in physiological saline. In *Brucella* broth incubated at 37°C for seven days, a moderate turbidity is produced, with a ropy or viscous sediment that cannot be uniformly resuspended. Older cultures may form a fine surface pellicle, which is easily disrupted. In serum-dextrose broth or well-buffered media, the ropy sediment or pellicle is often not produced. Cultures are always in the rough or mucoid phase on primary isolation. A smooth phase is not known. Cultures are not agglutinated by antisera monospecific for the A and M antigens, but do agglutinate with antiserum to the R antigen of *B. ovis*. Cross-reactions with the surface antigens of nonsmooth strains of other *Brucella* species also occur. Internal antigens are shared with smooth and nonsmooth strains of all *Brucella* strains. Metabolic activity is very similar to *B. suis* biovar 1, but urocanic acid is oxidized.

Pathogenic for the dog, producing chronic bacteremia and localizing granulomatous lesions. Epididymo-orchitis and prostatitis are produced in the male, and metritis, placentitis, and abortion are produced in the pregnant female. The infection is occasionally transmitted to humans; natural infections in other species have not been authenticated. Infection in guinea pigs and mice may be established by inoculation of large doses of organisms. The mouse retains the infection longer than the guinea pig.

The mol% G + C of the DNA is: 56 (T_m).

Type strain: RM6/66, ATCC 23365, NCTC 10854.

Additional Remarks: Genetic and other evidence suggests *B. canis* is a stable rough variant of *B. suis* biovar 1.

4. ***Brucella neotomae*** Stoenner and Lackman 1957, 947^{AL}
ne.o.to' mae. M.L. fem. n. *Neotoma*, *Neotoma lepida* generic name of the desert wood rat of the Western U.S.A (Thomas); M.L. fem. gen. n. *neotomae* of the desert wood rat, the host from which the organism was first isolated.

The morphological and cultural characteristics are as described for the genus. Other characteristics are indicated in Tables BXII.α.120 and BXII.α.121. Nonpathogenic for cattle, sheep, goats, and pigs. Not proven to be pathogenic for humans. Does not apparently produce disease in its natural host (the desert wood rat) and shows minimal pathogenicity for laboratory animals. Guinea pigs develop slight splenomegaly, and sometimes epididymo-orchitis or testicular abscesses following intraperitoneal inoculation. Small granulomatous lesions develop in the liver and spleen. Mice are more susceptible to infection, and lesions may be produced by frequently passaged strains.

The mol% G + C of the DNA is: 56–57 (T_m).

Type strain: 5K33, ATCC 23459; NCTC 10084.

5. ***Brucella ovis*** Buddle 1956, 351^{AL}
o'vis. L. gen. n. *ovis* of the sheep.

The morphological and cultural characteristics are as described for the genus. Although other species of *Brucella* stain red by modified Köster's stain, *B. ovis* stain blue. Other characteristics are listed in Tables BXII.α.120 and BXII.α.121; no biovars are recognized. L-alanine, L-aspartic acid, L-glutamic acid, adonitol, and DL-serine are oxidized. Cultures are known to exist only in the nonsmooth colonial phase. They do not agglutinate with antisera monospecific for A and M surface antigens, but are agglutinated by antisera to the R surface antigen of *B. ovis*. Cross-reactions occur with the surface antigens of nonsmooth strains of other *Brucella* species. Many of the internal antigens are shared with other *Brucella* species, irrespective of colonial phase.

Pathogenic for sheep, producing epididymo-orchitis in the male and placentitis and abortion in the pregnant female. Goats may be infected experimentally and subclinical infections may be produced in cattle, guinea pigs, rabbits, mice, and gerbils.

The mol% G + C of the DNA is: 57–58 (T_m).

Type strain: 63/290, ATCC 25840, NCTC 10512.

GenBank accession number (16S rRNA): L26168.

Additional Remarks: The *B. ovis* genome contains many more copies of the insertion sequence IS6501 than the genomes of the other nomenspecies.

6. ***Brucella suis*** Huddleson 1929, 12^{AL}
su'is. L. gen. n. *suis* of the pig.

The morphological and cultural characteristics are as described for the genus. Other characteristics of the species and its biovars are indicated in Tables BXII.α.120 and BXII.α.121. Biovars 1, 2, and 3 are naturally pathogenic for pigs. Biovar 2 also naturally infects hares. Strains resembling biovar 3 have also been isolated from various species of rodents. Biovar 4 is naturally pathogenic for reindeer (Davydov, 1961). Biovar 5 has only been isolated from rodents

(Corbel, 1982; 1988). All biovars, with the possible exception of biovar 2, are pathogenic for humans. Other species, including dogs, horses, and many species of rodents, may also be infected. In the natural hosts, generalized infections are produced, with localizing lesions, particularly in the genitalia. The testes, epididymides, and seminal vesicles are usually severely affected in the male. Metritis, placentitis, and abortion are produced in the pregnant female.

Pathogenic for laboratory animals including rabbits, guinea pigs, and mice. Splenomegaly and widespread granulomatous and suppurative lesions are produced. Biovars 1 and 3 are usually the most virulent, and heavy inocula may produce fatal infections in guinea pigs.

The mol% G + C of the DNA is: 56–57 (T_m).

Type strain: 1330, ATCC 23444, NCTC 10316.

GenBank accession number (16S rRNA): L26169.

Additional Remarks: ATCC 23444 is also the reference strain for biovar 1 (referred to as “American *suis*”). For biovar 2 (referred to as “Danish or European *B. suis*” by Thomsen, 1929), the reference strain is Thomsen (ATCC 23445; NCTC 10510). For biovar 3 (referred to as “American *melitensis*” or “dye-resistant *B. suis*” [Huddleson, 1957]), the reference strain is 686 (ATCC 23446; NCTC 10511). This biovar is unique in having a single chromosome of 3.2×10^6 base pairs. For biovar 4 (formerly “*Brucella rangiferi tarandi*” [Davydov, 1961]), the reference strain is 40 (ATCC 23447; NCTC 11364). For biovar 5 the reference strain is 513 (NCTC 11996). Biovar 3 is probably closest to the ancestral prototype of the genus. It is believed that the double chromosomes of the other nomenspecies and biovars arose because of rearrangements during replication.

Other Organisms

“*Brucella maris*”

The designation “*Brucella maris*” has been suggested for strains of *Brucella* isolated from marine mammals. However, it is now clear that these comprise at least four distinct groups, according to conventional typing and host range, and at least seven types based on restriction fragment polymorphism of the *omp2a*, *2b*, and *omp25* genes. Three new nomenspecies have been proposed to cover the known variants. These are “*B. phocae*” with two biovars, associated with seals, “*B. phocoenae*” and “*B. delphini*” with one biovar each, isolated from porpoises and dolphins, respectively. All strains oxidize D-glutamic acid, ribose, and xylose, whereas none oxidize L-alanine, L-aspartic acid, L-arginine, L-ornithine, or L-lysine. Strains of “*B. phocae*” biovars 1 and 2 oxidize D-erythritol but only biovar 2 oxidizes urocanic acid. Both “*B. phocoenae*” and “*B. delphini*” oxidize D-galactose; the former oxidizes D-erythritol but not urocanic acid, whereas the latter shows the reverse pattern. Nearly all strains are lysed by Bk₂ phage and none are lysed by phage R/Cat-RTD. Strains of “*B. phocae*”

biovar 2 and “*B. delphini*” are lysed by Tb and Fi phages at RTD, whereas “*B. phocae*” biovar 1 and “*B. phocoenae*” are not. Only strains of “*B. phocae*” biovar 2 are usually lysed by Wb phage. There is some variation in phage sensitivity between strains within putative nomenspecies, suggesting that phage typing could be applicable for subgrouping.

Strains of both “*B. phocae*” biovars require CO₂, but the other nomenspecies do not. None produce H₂S, but all are urease positive and grow in the presence of basic fuchsin and thionine. European isolates obtained to date have been A-antigen dominant, but North American strains dominant for M antigen have also been described. The latter may correspond to an additional nomenspecies. Pathogenicity for natural host species has not been clearly defined; subclinical infection seems the usual outcome. Potentially pathogenic for humans (Brew et al., 1999).

The status of this group requires resolution. It is clear that it represents a novel subdivision within the genus, but with considerable internal diversity.

Genus II. *Mycoplana* Gray and Thornton 1928, 82^{AL} emend. Urakami, Oyanagi, Araki, Suzuki and Komagata 1990d, 439

TEIZI URAKAMI AND PAUL SEGERS

My.co.pla' na. Gr. *mykes* fungus; Gr. *plane* a wandering; M.L. fem. n. *Mycoplana* fungus wanderer.

Cells are **curved or irregular rods** with rounded ends, $0.5\text{--}0.8 \times 2.0\text{--}3.0 \mu\text{m}$. Occur singly or rarely in pairs. The cells form **branching filaments** prior to fragmentation into irregular rods. Gram negative and nonsporeforming. **Motile by means of peritrichous flagella.**

Colonies are white to light yellow.

Aerobic, with a **strictly respiratory** type of metabolism; not fermentative. Grow well on standard culture media, between pH 6 and 8, at 30°C, but not in medium containing 3% NaCl and not at 42°C. The methyl red test is negative. Indole and hydrogen sulfide are not produced. Hydrolysis of gelatin and starch does not occur. Ammonia is produced. Denitrification is negative and litmus milk is not changed. Acid is produced only oxidatively from sugars. Urease and oxidase are produced. A water-soluble fluorescent pigment is not produced on King A or King B media. The cells **accumulate granules of poly-β-hydroxybutyrate.** Occur

in soil. The major fatty acids are octadecenoic acid (C_{18:1}), in large amounts, hexadecanoic acid (C_{16:0}), and hexadecenoic acid (C_{16:1}). The major hydroxy fatty acid is C_{14:0} 3OH. They contain Q-10 as major ubiquinone.

The mol% G + C of the DNA is: 63–65.

Type species: *Mycoplana dimorpha* Gray and Thornton 1928, 82 emend. Urakami, Oyanagi, Araki, Suzuki and Komagata 1990d, 440.

FURTHER DESCRIPTIVE INFORMATION

The genus *Mycoplana* as emended and described by Urakami et al. (1990d) is paraphyletic, as shown by rRNA gene sequence analysis (Abraham et al., 1999, see also the chapter on *Brevundimonas*). *Mycoplana dimorpha* and *Mycoplana ramosa* belong to a separate lineage within the family *Brucellaceae*, whereas the type strains of *Mycoplana bullata* and *Mycoplana segnis* belong to the

family *Caulobacteraceae*. Furthermore, *M. bullata* was shown to belong to the genus *Brevundimonas* and *M. segnis* was reclassified as *Caulobacter segnis* (Abraham et al., 1999). Since the last two species belong to other genera, the authentic genus *Mycoplana* contains only the species *M. dimorpha* (the type species) and *M. ramosa*. Therefore, in this chapter, a corresponding restrictive description is given for the genus. *M. bullata* and *Caulobacter segnis* (*M. segnis*) are described in the chapters on the genera *Brevundimonas* and *Caulobacter*, respectively, the genera in which they are presently classified.

Colonies of the genus *Mycoplana* are not mucous. Voges–Proskauer and catalase tests are positive. Ammonium salts, urea, peptone, Casamino acids, and, for most strains, nitrate are utilized as nitrogen and or carbon sources. Biotin or thiamine is required for growth. They utilize as sole source of carbon L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, D-sorbitol, D-mannitol, glycerol, L-glutamic acid, L-aspartic acid, acetic acid, putrescine, spermidine, and spermine. Lactose, trehalose, inositol, soluble starch, citric acid, formic acid, methanol, ethanol, butanol, monomethylamine, methane, and butane are not utilized.

In an extensive comparative study between members of the *Rhizobiaceae*, assimilation of 147 carbohydrates by the type strains of *M. dimorpha* and *M. ramosa* (NCIB 9440) was shown (de Lajudie et al., 1994). In this study, the assimilation of maltose, sucrose and succinate was shown to be negative for *M. dimorpha*, and it was demonstrated that the assimilation of ribose, L-sorbose, inositol, 2-ketogluconate, butyrate, DL-3-hydroxybutyrate, L-valine, betaine, spermine, and glucosamine can be used to distinguish the two species *M. dimorpha* and *M. ramosa*.

In the emended description of *Mycoplana* (Urakami et al., 1990d), strains of *M. dimorpha* and *M. ramosa* were shown to produce oxidatively acid from L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, D-sorbitol, D-mannitol, and glycerol, but not from maltose, sucrose, lactose, trehalose, inositol, and soluble starch. Hydrolysis of gelatin and starch was not observed. Contradicting the results of Gray and Thornton (1928), *Mycoplana* strains produced no acid from carbohydrates, and hydrolysis of starch and gelatin liquefaction were variable features.

The ability to decompose aromatic compounds, such as phenol and *m*-cresol, is included in the original description (Gray and Thornton, 1928).

Mycoplana strains that are diazotrophic under microaerobic conditions have been described. Several *M. dimorpha* and *M. bullata* strains showed nitrogenase activity and ^{15}N enrichment when grown on N-free media (Pearson et al., 1982).

Q-10 is the major ubiquinone; also detected were small amounts of Q-9 and traces of Q-11.

ENRICHMENT AND ISOLATION PROCEDURES

Isolation of *Mycoplana* strains was originally described by Gray and Thornton (1928): inoculate 100 ml of a solution of mineral salts, to which 0.05–0.1% phenol or 0.05% *m*-cresol is added, with soil (0.5–1 g). If growth occurs, make transfers into fresh flasks of the same medium. After further plating and purification on an agar medium, *Mycoplana* strains can be isolated.

MAINTENANCE PROCEDURES

Cultures can be maintained on most common media. Abundant growth occurs in nutrient broth, PYG broth, and peptone water, and on these solid media, when grown at 30°C in air. Cultures

can be stored at room temperature or in the refrigerator, but should be transferred weekly, although longer storage in a refrigerator is possible.

Cultures may be stored for many years by lyophilization, freezing at -80°C , or in liquid nitrogen. Cryoprotective agents such as 10% glycerol or DMSO should be added to cultures before freezing, and heavy cell concentrations should be used.

DIFFERENTIATION OF THE GENUS *MYCOPLANA* FROM OTHER GENERA

Mycoplana belongs phylogenetically to the family *Brucellaceae*, which also contains the genera *Brucella* and *Ochrobactrum*. *Mycoplana* can easily be differentiated from the other genera by the ability to form branching filaments (Fig. BXII.α.140), motility (*Brucella* is nonmotile), accumulation of granules of poly-β-hydroxybutyrate, utilization and acid production from several carbohydrates (Holmes et al., 1988; de Lajudie et al., 1994; see Table BXII.α.123 in the chapter on *Ochrobactrum*), and the hydrolysis of L-isoleucyl-β-naphthylamide and L-prolyl-β-naphthylamide hydroxychloride (API-ZYM test, Holmes et al., 1988).

The former *Mycoplana* species *M. bullata* and *C. segnis* can be distinguished from the authentic *Mycoplana* species *M. dimorpha* and *M. ramosa* by their phylogenetic position, mucoid colonies, requirement for growth of special unknown growth factors or peptone, no production of acid from several carbohydrates, occurrence of $\text{C}_{12:0} 3\text{OH}$, and only small amounts of $\text{C}_{14:0} 3\text{OH}$ and larger amounts of hexadecanoic acid ($\text{C}_{16:0}$), and the higher mol% G + C content (66–68), (Urakami et al., 1990d).

Mycoplana strains resemble *Methylobacterium*, *Xanthobacter*, and *Oerskovia* strains based on branching filament formation and/or a few chemotaxonomic properties. One can easily differentiate those taxa by their different phylogenetic position, Gram stain, the fine structure of the cells, colony color, utilization of methanol, and hydroxy fatty acid composition (Urakami et al., 1990d).

TAXONOMIC COMMENTS

In 1928, Gray and Thornton created the genus *Mycoplana* for branching bacteria isolated from soil and able to decompose aromatic compounds. They classified this new genus with two species, *M. dimorpha* (the type species) and *M. bullata*, in the family *Mycobacteriaceae*.

The ability of the strains to form branching filaments prior to breaking up into motile rods has led several workers to place the genus *Mycoplana* in the order *Actinomycetales*, although they have a cell wall of the Gram-negative type, containing *meso*-DAP and numerous amino acids (Sukapure et al., 1970; Cross and Goodfellow, 1973; Lechevalier and Lechevalier, 1981a, c). In different editions of *Bergey's Manual of Determinative Bacteriology*, the genus was placed in the family *Mycobacteriaceae* or in the family *Pseudomonadaceae* (Breed, 1957) or even, in the eighth edition, left out (Buchanan and Gibbons, 1974). In the first edition of *Bergey's Manual of Systematic Bacteriology*, it was mentioned in the section "Nocardioform Actinomycetes", as a comment, supplementary to the genus *Oerskovia* (Lechevalier and Lechevalier, 1981b).

Mycoplana, *M. dimorpha*, and *M. bullata* are valid names since they appear on the Approved Lists of Bacterial Names (Skerman et al., 1980).

The genus description was emended in 1990, and two new species, *M. ramosa* and *M. segnis*, were proposed by Urakami et

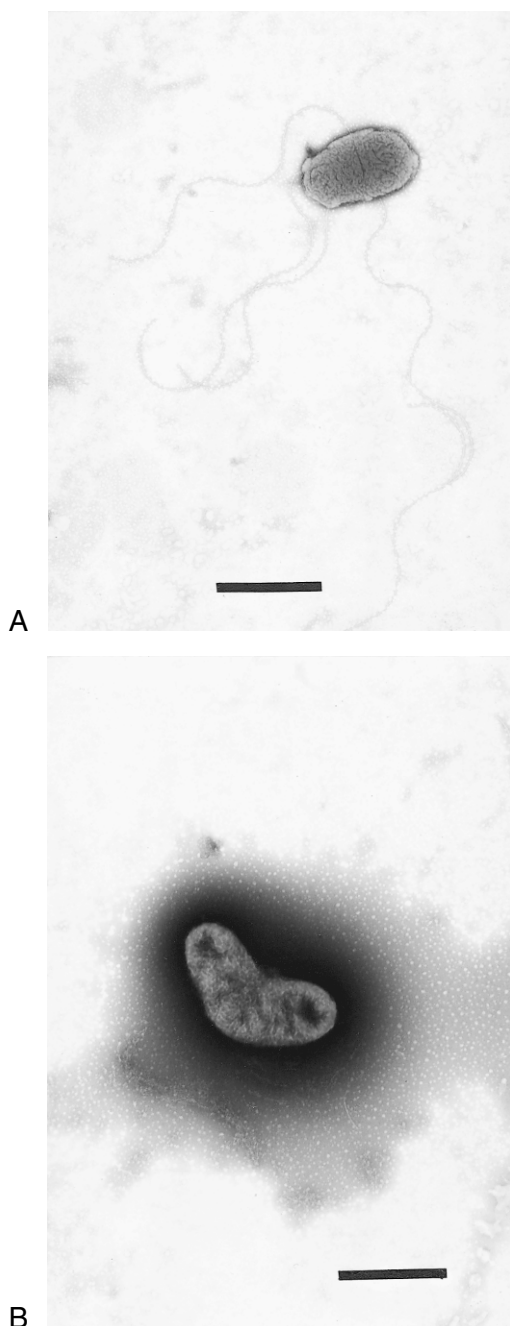


FIGURE BXII.α.140. Negatively stained cells of *Mycoplana dimorpha* ATCC 4279^T (Bar = 1 μm). (A) peritrichous flagella; (B) exhibiting branching.

al. (1990d). In a polyphasic approach, they recharacterized and clarified the authenticity of the type strains of *M. dimorpha* and *M. bullata* from different origins as well as a few other *Mycoplana* strains. From the strains of *M. bullata*, received from different culture collections, only ATCC 4278^T (IFO 13290^T) was, based

on strain authenticity investigations, identified as the original type strain for *M. bullata*. *M. bullata* strain NCIB 9440, supposedly originating from the same source, differs from this species. A new species, *M. ramosa*, was proposed for this strain. *M. segnis* was proposed as a new taxon with two strains.

Recent rRNA similarity studies showed that the genus *Mycoplana* is paraphyletic (Yanagi and Yamasato, 1993; Abraham et al., 1999; see Fig. BXII.α.122 in the chapter on *Brevundimonas*). With two species, *M. dimorpha* and *M. ramosa*, *Mycoplana* constituted a separate branch, closely related to *Brucella* and *Ochrobactrum* in the family *Brucellaceae*. 16S rRNA gene sequencing studies showed *M. bullata* to cluster in the *Caulobacteraceae* as a separate branch within the emended genus *Brevundimonas* (Abraham et al., 1999; see chapter *Brevundimonas*). It can be considered as a *Brevundimonas* species. *M. segnis*, a member of the same rRNA family, is more closely related to the type species of *Caulobacter* and has been reclassified as *Caulobacter segnis* (see Fig. BXII.α.122 and Table BXII.α.107 in the chapter *Brevundimonas*; Abraham et al., 1999). The unexpectedly high level of interrelationship of both former *Mycoplana* species with the emended genera *Brevundimonas* and *Caulobacter* was also confirmed by polar lipid and fatty acid analysis, mol% G + C content, and phenotypic characterization.

Sensitivity to the rhizobial peptide antibiotic trifolitoxin was found in *Mycoplana* species and in organisms of related genera (Triplett et al., 1994).

Lipopolysaccharides were isolated and characterized from *M. dimorpha*, *M. ramosa*, *M. bullata*, and *C. segnis* (Tharanathan et al., 1993). Similarities in chemical composition between related organisms could be shown. *M. ramosa* and *M. dimorpha* expressed ladder-like patterns on DOC-PAGE, albeit weakly, indicating some S-type lipopolysaccharides and lipid A of the D-glucosamine type. *M. bullata* and *M. segnis* showed an R-type on DOC-PAGE and had lipid As of the lipid A-DAG-type, which exclusively contained 2,3-diamino-2,3-dideoxy-D-glucose as lipid A sugar (Tharanathan et al., 1993).

"*Mycoplana rubra*" (Devries and Derx, 1953), a pink methylamine-utilizing bacterium was deposited in the National Collection of Industrial Bacteria (Aberdeen, UK). This organism is now classified in the facultative methylotrophic genus *Methylobacterium* (Urakami and Komagata, 1984; Bousfield and Green, 1985).

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *MYOPLANA*

Characteristics useful in distinguishing the two authentic *Mycoplana* species *M. dimorpha* and *M. ramosa* are the ability of the first species to assimilate maltose, sucrose, and succinic acid, and its requirement of biotin for growth. *M. ramosa* requires thiamine

for growth (Urakami et al., 1990d). Differences in the utilization and acid production from other carbohydrates between the two species have also been shown (Holmes et al., 1988; de Lajudie et al., 1994).

List of species of the genus *Mycoplasma*

1. ***Mycoplasma dimorpha*** Gray and Thornton 1928, 98^{AL} emend. Urakami, Oyanagi, Araki, Suzuki and Komagata 1990d, 440.

di.mo'pha. Gr. adj. *dimorpha* two forms.

Morphology and characteristics are as given for the genus.

Reduction of nitrate is variable. Biotin is required for growth. Maltose, sucrose, and succinic acid are also assimilated.

The mol% G + C of the DNA is: 64–65 (HPLC).

Type strain: ATCC 4279, IFO 13291, NCIB 9439, LMG 4061.

2. ***Mycoplasma ramosa*** Urakami, Oyanagi, Araki, Suzuki and Komagata 1990d, 441^{VP}
ra.mo'sa. M.L. *ramosa* ramous, ramose, branched, branch-like.

Morphology and characteristics are as given for the genus.

Reduction of nitrate and the Voges–Proskauer test are weakly positive. Thiamine is required for growth. Maltose, sucrose, and succinic acid are not assimilated.

The mol% G + C of the DNA is: 64 (HPLC).

Type strain: ATCC 49678, NCIB 9440, IFO 15249, LMG 3026.

Genus III. ***Ochrobactrum*** Holmes, Popoff, Kiredjian and Kersters 1988, 412^{VP}

BARRY HOLMES

O.chro.bac'trum. Gr. adj. *ochros* pale, colorless; Gr. neut. n. *baktron* a staff or stick; M.L. neut. n.

Ochrobactrum a colorless rod.

Rods with parallel sides and rounded ends, typically 1.0×1.5 – $2.0 \mu\text{m}$; shorter oval forms, 1.0 – $1.5 \mu\text{m}$ in length, may occur. Cells usually occur singly. **Gram negative.** **Motile** by means of peritrichous flagella. **Obligately aerobic**, having a strictly **respiratory** type of metabolism. Optimal growth temperature in the range 20 – 37°C . Colonies on nutrient agar are smooth, low convex, and translucent (opaque and mucoid, quickly becoming confluent in *O. tritici*). Chemoorganotrophic, using a variety of amino acids, organic acids, and carbohydrates as carbon sources. Acid is produced from glucose, arabinose, ethanol, fructose, rhamnose, and xylose. **Oxidase positive.** **Catalase positive.** Indole is not formed. **Nitrates usually reduced.** Growth occurs on MacConkey agar. Urease usually positive. Ornithine decarboxylase (ODC) negative. Occur in human clinical specimens, also in soil samples and wheat roots. Parameters of DNA–rRNA hybrids indicate that *Ochrobactrum* belongs to the *Brucella* branch within rRNA superfamily IV. 16S rRNA gene sequence analysis places *Ochrobactrum* in the group of bacteria known as the α -2 subclass of the *Proteobacteria*. At the suprageneric level, *Ochrobactrum* is related to *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Agrobacterium*.

The mol% G + C of the DNA is: 56–59.

Type species: ***Ochrobactrum anthropi*** Holmes, Popoff, Kiredjian and Kersters 1988, 412.

FURTHER DESCRIPTIVE INFORMATION

Cells of *Ochrobactrum* grown on nutrient agar are monomorphic straight rods and have rounded ends. The usual cell dimensions are 1.0×1.5 – $2.0 \mu\text{m}$. Shorter oval forms, 1.0 – $1.5 \mu\text{m}$ in length, may occur.

Sudanophilic bodies have not been detected with the use of Sudan Black B.

After incubation for 24 h at 37°C on nutrient agar, colonies are <0.5 mm in diameter, circular, low convex, smooth, moist, glistening, translucent, and butyrous, and have an entire edge; pigment is not produced.

Growth at 20°C and 37°C is equally good. Few strains grow at 42°C , and while clinical strains have not been reported to grow at 5°C , environmental isolates will grow at 4°C .

Immunological cross-reactions between *O. anthropi* and *Bru-*

cella spp. are apparent in animals infected by *Brucella* spp. Pulsed-field gel electrophoresis and polymerase chain reaction genome fingerprinting based on repetitive chromosomal sequences (rep-PCR) have been applied to the typing of strains of *O. anthropi* (van Dijck et al., 1995). Two chromosomes are present.

Clinical strains have been reported as resistant to ampicillin, amoxicillin + clavulanic acid, aztreonam, cefamandole, cefonicid, cefoperazone, cefoxitin, cefsulodin, ceftazidime, cefuroxime, cephalothin, chloramphenicol, erythromycin, fosfomycin, kanamycin, mezlocillin, pipemidic acid, piperacillin, pristnamycin, streptomycin, and ticarcillin. Strains are said to be sensitive to amikacin, cefoperazone, ceftriaxone, ciprofloxacin, gentamicin, imipenem, netilmicin, nalidixic acid, pefloxacin, rifampicin, tetracycline, and vancomycin; most are also susceptible to moxalactam. However, different authors find different results with cefotaxime, colistin, newer fluoroquinolones, and trimethoprim-sulfamethoxazole. Monotherapy with an appropriate aminoglycoside or an appropriate β -lactam (such as ceftriaxone) has yielded a good clinical response in the treatment of bacteremia. However, despite determining *in vitro* susceptibility to imipenem in initial isolates, treatment of two patients with this agent failed to eradicate the organism (Kern et al., 1993).

In humans, *O. anthropi* has been reported from various clinical specimens, mostly as a cause of bacteremia but also of endophthalmitis, meningitis, necrotizing fasciitis, pancreatic abscess, and puncture wound osteochondritis of the foot. *O. anthropi* is apparently often community acquired and can be pathogenic in critically ill or immunocompromised patients, with or without indwelling catheters; although it can produce clinically significant infections, it appears to be of relatively low virulence. The organism has also been reported from activated sludge, biofilm in water supply lines, soil, and the termite gut. *O. intermedium* has also been isolated from human blood and soil. *O. grignonense* and *O. tritici* have so far been reported predominantly from soil and wheat roots, respectively.

ENRICHMENT AND ISOLATION PROCEDURES

Special procedures are not required for the isolation of *Ochrobactrum* strains.

MAINTENANCE PROCEDURES

Stock cultures can be maintained for several months on a slant of Dorset egg medium (Barrow and Feltham, 1993) in a metal screw-capped bijou bottle stored at 4°C. Cultures can be maintained for longer periods by suspending growth from an 18-h-old agar slant culture in defibrinated rabbit blood, transferring to a small tube (capped or plugged), and freezing in a mixture of dry ice and alcohol prior to storage at -50°C. Strains of *Ochrobactrum* may also be preserved by lyophilization.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

A major feature of *Ochrobactrum* strains is their motility by means of from two to several subpolar peritrichous flagella. Motility should be determined in hanging-drop preparations after overnight growth in nutrient broth, incubated at either 37°C or room temperature (18–22°C). The peritrichous nature of the flagella and their lateral attachment are best demonstrated by electron microscopy.

The saccharolytic ability of strains must be determined in a medium with either a low peptone content, such as Hugh and Leifson medium, or with no peptone, such as ammonium salt sugars. In high peptone-content media, alkali production from the peptone will mask the acid produced by oxidation of the carbohydrate.

DIFFERENTIATION OF THE GENUS *OCHROBACTRUM* FROM OTHER GENERA

Characteristics for the differentiation of *Ochrobactrum* from closely related and phenotypically similar genera are given in Table BXII.α.123.

TAXONOMIC COMMENTS

Holmes et al. (1988) found two DNA hybridization groups among the 56 strains for which they proposed the name *O. anthropi*. The majority of strains tested grouped around the proposed type strain of the species (NCTC 12168 LMG 3331), with an average relative binding ratio of 80.5% ± 7.2%. A second, smaller group of strains formed around reference strain NCTC 12171 (= LMG 3301), with an average relative binding ratio of 87% ± 11%. Two further reference strains occupied a somewhat separate position. The two DNA hybridization groups hybridized at an average degree of relative binding of 45% ± 4%. The major hybridization group of 32 strains could be divided into biovars A and D (corresponding to the *Achromobacter* Groups A and D of Holmes and Dawson 1983). The DNA-DNA hybridization data, however, clearly demonstrated that the three strains constituting

biovar D showed a high degree of DNA relatedness (≥90%) to the strains of biovar A, despite having phenotypic differences. The second DNA hybridization group of five strains contained two which formed biovar C (corresponding to the *Achromobacter* Group C of Holmes and Dawson 1983). However, the remaining three strains of the second DNA hybridization group (including LMG 3301) corresponded to biovar A, as did the two strains which occupied an intermediate hybridization position.

In summary, Holmes et al. (1988) found *O. anthropi* to be phenotypically homogeneous except for five strains; three of these represented a separate biovar but were genotypically indistinguishable from the major group of strains. *O. anthropi* was also genotypically homogeneous, except for seven strains, but only two of these could be distinguished phenotypically from the major group of strains. Holmes et al. (1988) proposed recognition of their group of strains as a single species. This view was not accepted by Velasco et al. (1998), who re-examined LMG 3301 (= NCTC 12171) and some additional strains, and found them more closely related to *Brucella* than to the group containing the type strain of *O. anthropi*, NCTC 12168 (= LMG 3331). The two groups could be differentiated by 16S rRNA gene sequence analysis and by 16S rRNA PCR with *Brucella*-specific primers. It was specifically recommended that distinct genomic groups not be proposed as named species until adequate phenotypic characters for differentiation from related genomic groups (Wayne et al., 1987) are available. Despite the fact that the two groups differed only in susceptibility to colistin, Velasco et al. (1998) considered this sufficient to propose a separate species, *O. intermedium*, for the group containing LMG 3301 (= NCTC 12171).

Subsequently, Lebuhn et al. (2000) studied isolates of *Ochrobactrum* from soil samples and wheat roots. They described two new species, *O. grignonense* and *O. tritici*, and found additional phenotypic characters to distinguish *O. intermedium* from *O. anthropi*. They also countenanced the future transfer of *O. intermedium* to a new genus, in order to yield monophyletic lineages that correspond to results from genotyping.

FURTHER READING

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TABLE BXII.α.123. Differentiation of *Ochrobactrum* from closely related and phenotypically similar genera^a

Characteristic	<i>Ochrobactrum</i>	<i>Agrobacterium</i>	<i>Alcaligenes</i>	<i>Brucella</i>	<i>Mycoplana</i>	<i>Phyllobacterium</i>	<i>Rhizobium</i>
<i>Acid from:</i>							
Ethanol	+	6/7	14/19	—	—	—	—
Fructose	+	+	—	—	+	+	+
Raffinose	—	+	—	—	—	—	1/3
<i>Utilization of:</i>							
Glycine	+	—	13/19	—	—	—	—
Succinate	+	+	+	—	—	+	+
Rhamnose	+	+	—	—	—	+	+
Mannitol	+	+	—	—	+	+	+
Sorbitol	+	+	—	—	+	+	+
D-Arabitol	+	+	—	—	+	+	+
<i>Hydrolysis of:</i>							
L-Isoleucyl-β-naphthylamide	—	—	—	—	+	—	—

^aSymbols: +, all strains tested were positive; —, all strains tested were negative. x/x, number of strains positive/number tested. Data from Holmes et al. (1988).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *OCHROBACTRUM*

Phenotypic differentiation among the four named species of *Ochrobactrum* is given in Table BXII.α.124.

List of species of the genus *Ochrobactrum*

1. ***Ochrobactrum anthropi*** Holmes, Popoff, Kiredjian and Kersters 1988, 412^{VP}
an.thro'pi. Gr. n. *anthropos* a human being; N.L. gen. n. *anthropi* of a human being.

The characteristics are as described for the genus. Additional characteristics are listed in Table BXII.α.125. The species was originally divided into three biovars A, C, and D, corresponding to the *Achromobacter* groups A, C, and D, respectively, of Holmes and Dawson (1983). On genomic grounds, however, only biovars A and D can be retained for *O. anthropi*; strains of biovar A differ from those of biovar D in producing acid from adonitol and sucrose, also in failing to utilize L-arabitol.

The mol% G + C of the DNA is: 56–59 (T_m).

Type strain: NCTC 12168, CIP 82.11, CIP 14970, DSM 6882, IAM 14119, LMG 3331.

GenBank accession number (16S rRNA): D12794.

2. ***Ochrobactrum grignonense*** Lebuhn, Achouak, Schlöter, Berge, Meier, Barakat, Hartmann and Heulin 2000, 2221^{VP}
gri.gno.nen'se. Fr. n. *Grignon* a location in France; L. neut. suff. *-ense* indicating provenance; N.L. neut. adj. *grignonense* pertaining to Grignon, region from which the strains were isolated.

The characteristics are as described for the genus. Additional characteristics are listed in Table BXII.α.125. The principal diagnostic phenotypic characteristics of *O. grignonense* are production of acid from arabinose and melibiose, assimilation of mannose but not maltose, and utilization of malonic acid but not adonitol or D-glucosaminic acid.

The mol% G + C of the DNA is: 58 (T_m).

Type strain: OgA9a, LMG 18954, DSM 13338.

GenBank accession number (16S rRNA): AJ242581.

3. ***Ochrobactrum intermedium*** Velasco, Romero, López-Goñi, Leiva, Díaz and Moriyón 1998, 767^{VP}
in'ter.me'dium. L. neut. adj. *intermedium* of intermediate position.

The characteristics are as described for the genus. Additional characteristics are listed in Table BXII.α.125. There are few phenotypic tests to differentiate *O. intermedium* from *O. anthropi*, but strains of the former are said to differ from those of the latter in being resistant to both colistin and polymyxin B, in failing to produce urease, and in failing to utilize quinic acid.

The mol% G + C of the DNA is: 58 (T_m).

Type strain: NCTC 12171, LMG 3301.

GenBank accession number (16S rRNA): U70978.

4. ***Ochrobactrum tritici*** Lebuhn, Achouak, Schlöter, Berge, Meier, Barakat, Hartmann and Heulin 2000, 2222^{VP}
tri'ti.ci. M.L. gen. n. *tritici*, from *Triticum*, generic name for wheat, from which the strains were isolated.

The characteristics are as described for the genus. Additional characteristics are listed in Table BXII.α.125. The principal diagnostic phenotypic characteristics of *O. tritici* are assimilation of gluconate, and utilization of sebamic acid but not γ-hydroxybutyric acid.

The mol% G + C of the DNA is: 59 (T_m).

Type strain: SCII24, LMG 18957, DSM 13340.

GenBank accession number (16S rRNA): AJ242584.

TABLE BXII.α.124. Differentiation of *Ochrobactrum* species^a

Characteristics	<i>O. anthropi</i>	<i>O. grignonense</i>	<i>O. intermedium</i>	<i>O. tritici</i>
<i>Acid from:</i>				
Arabinose	—	± to +	—	—
Melibiose	—	+	—	—
<i>Antimicrobial susceptibility:</i>				
Colistin	S/I	R	R	S
Polymyxin B	S	R	R	S
<i>Assimilation of:</i>				
Maltose (48 h)	+	—	+	+
Mannose (24 h)	—	+	—	—
Urease production (24 h)	+	—	—	+
<i>Utilization of:</i>				
Adonitol	+	—	+	+
Cellobiose	+	— to ±	+	—
Gentiobiose	+	— to ±	+	—
Malonic acid	—	+	—	—
Quinic acid	+	—	—	—
D-Glucosaminic acid	+	—	+	+
D-Trehalose	+	—	+	+
γ-Hydroxybutyric acid	+	+	+	—

^aFor symbols see standard definitions; ±, borderline reaction; I, intermediate; R, resistant; S, susceptible. Data from Lebuhn et al., 2000.

TABLE BXII.α.125. Additional characteristics of the species of the genus *Ochrobactrum*^a

Characteristics	<i>O. anthropi</i>	<i>O. grignonense</i>	<i>O. intermedium</i>	<i>O. tritici</i>
Arginine dihydrolase	—	—	—	—
<i>Assimilation of:</i>				
Adipate	—	—	—	—
Arabinose (48 h)	+	+	+	+
Citrate (24 h)	—	+	+	— to ±
Gluconate (48 h)	— to ±	— to ±	— to ±	+
Glucose (48 h)	+	+	+	+
Mannitol	—	—	—	—
Phenylacetate	—	—	—	—
Chloramphenicol susceptibility	R	I/S	R	I
Denitrification	+	+	+	+
Fermentation of glucose	—	—	—	—
Gelatin hydrolysis	—	—	—	—
H ₂ S production	—	—	—	—
Indole production	—	—	—	—
Lysine decarboxylase	—	—	—	—
Ornithine decarboxylase	—	—	—	—
Production of β-galactosidase (ONPG and PNPG)	—	—	—	—
Tryptophan deaminase	—	—	—	—
<i>Utilization of:</i>				
Citrate (24 h)	—	—	—	—
Citrate (48 h)	+	+	+	+
Bromosuccinic acid	+	+	+	+
Glycerol	± to +	+	—	—
Glycyl-L-aspartic acid	+	+	+	+
Glycyl-L-glutamic acid	+	+	+	+
Hydroxy-L-proline	+	+	+	+
Inosine	+	+	+	+
Maltose	+	+	+	+
Monomethyl succinate	+	+	+	+
Phenylethylamine	—	—	—	—
Psicose	+	+	+	+
Sebacic acid	— to ±	— to ±	—	+
Succinic acid	+	+	+	+
Turanose	+	+	+	+
Tween 40	+	+	+	+
Tween 80	+	+	+	+
Uridine	± to +	+	— to ±	— to ±
Xylitol	— to ±	±	+	—
α-D-Glucose	+	+	+	+
α-Ketobutyric acid	+	+	+	+
α-Ketoglutaric acid	+	+	+	+
β-Hydroxybutyric acid	+	+	+	+
D-Alanine	+	+	+	+
D-Arabitol	+	+	+	+
D-Fructose	+	+	+	+
D-Galactose	+	+	+	+
D-Mannose	+	+	+	+
DL-α-Glycerol phosphate	± to +	± to +	—	— to ±
DL-Carnitine	+	— to ±	+	— to ±
DL-Lactic acid	+	+	+	+
DL-Erythritol	+	+	+	+
L-Alanine	+	+	+	+
L-Alanyl-glycine	+	+	+	+
L-Arabinose	+	+	+	+
L-Asparagine	+	+	+	+
L-Aspartic acid	+	+	+	+
L-Fucose	+	+	+	+
L-Glutamic acid	+	+	+	+
L-Histidine	+	+	+	+
L-Leucine	+	+	+	+
L-Ornithine	+	+	+	+
L-Phenylalanine	—	—	—	—
L-Proline	+	+	+	+
L-Rhamnose	+	+	+	+
L-Serine	+	+	+	+
L-Threonine	+	+	+	+
m-Inositol	+	+	+	+
γ-Aminobutyric acid	+	+	+	+

^aFor symbols see standard definitions; ±, borderline reaction; I, intermediate; R, resistant; S, susceptible. Data from Lebuhr et al., 2000.

Family IV. *Phyllobacteriaceae* fam. nov.

JORIS MERGAERT AND JEAN SWINGS

Phyl.lo.bac.teri.a'ce.ae. M.L. neut. n. *Phyllobacterium* type genus of the family; -aceae ending to denote family; M.L. fem. pl. n. *Phyllobacteriaceae* the *Phyllobacterium* family.

Rod-shaped, ovoid, or reniform cells when cultured *in vitro*. **Non-sporeforming.** **Gram negative.** **Aerobic.** Cells cultured *in vitro* are motile by means of polar, subpolar, or lateral flagella. Strains grow well on complex solid media at 28°C. **Occur in leaf nodules and the rhizosphere of higher plants.**

The mol% G + C of the DNA is: 60–62 (De Smedt and De Ley, 1977).

Type genus: *Phyllobacterium* (ex Knösel 1962) Knösel 1984b, 356 (Effective publication: Knösel 1984a, 254.)

TAXONOMIC COMMENTS

The family presently contains seven genera. Based on 16S rRNA gene sequence analysis the type genus, *Phyllobacterium*, is closely related phylogenetically to the genus *Mesorhizobium*. Together these genera form a separate branch in the *Alphaproteobacteria* and are phylogenetically related to the genera *Rochalimaea*, *Mycoplana*, *Bartonella*, *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Allo-rhizobium*,* *Ochrobactrum*, and *Brucella* (Yanagi and Yamasato, 1993; de Lajudie et al., 1994, 1998a, b; Jarvis et al., 1997). Apart from 16S rRNA gene similarities, there are as yet no other arguments available to unite the genera *Mesorhizobium* and *Phyllobacterium* in a single family. Both genera are very different, not only on the basis of habitat, plant interactions, and biochemical characteristics, but also chemotaxonomically. By analysis of total cellular fatty acid composition, the genera *Phyllobacterium*, *Mesorhizobium*, and members of the *Agrobacterium*–*Rhizobium*–*Sinorhi-*

zobium group form clearly separated entities, as exemplified in Fig. BXII.α.141 (see also Table BXII.α.126, and Jarvis et al., 1996). Unlike *Mesorhizobium* and other rhizobia, *Phyllobacterium* shows no evidence of nitrogen fixation (Lersten and Horner, 1976; Van Hove, 1976), and the genus lacks the *nif* HFK-like genes (Lambert et al., 1990), which occur in all the classical nitrogen-fixing bacteria.

Some differential features permitting differentiation of *Phyllobacterium* and *Mesorhizobium* are given in Table BXII.α.126.

ACKNOWLEDGMENTS

The authors are indebted to the Bijzonder Onderzoeksfonds (Belgium) for personnel grants, and to Margo Cnockaert for technical assistance.

TABLE BXII.α.126. Characteristics differentiating *Phyllobacterium* and *Mesorhizobium*^a

Characteristic	<i>Phyllobacterium</i>	<i>Mesorhizobium</i>
Assimilation of: ^b		
Lactose	–	+
<i>n</i> -Valerate, aconitate,	+	–
<i>p</i> -hydroxybenzoate, isovalerate		
Nitrogen fixation	–	+
Occurs in root nodules	–	+
Occurs in leaf nodules	+	–
Fatty acids in whole cell extracts: ^c		
C _{13:0} iso 3OH: C _{17:0} iso	–	+
C _{16:0} 3OH: C _{18:1} 2OH: C _{20:0}	+	–

^aFor symbols, see standard definitions.

^bData from de Lajudie et al. (1994).

^cFor strains investigated and methods used: see Figure BXII.α.141; Methods used according to Jarvis et al. (1996).

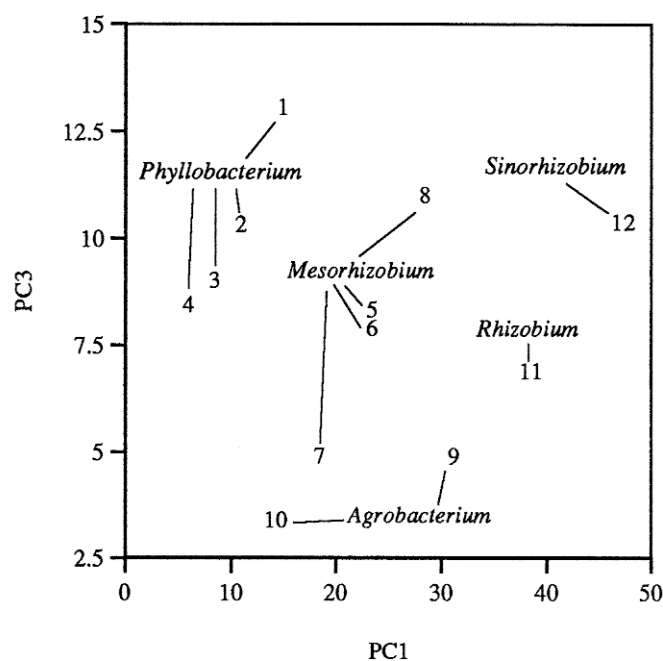
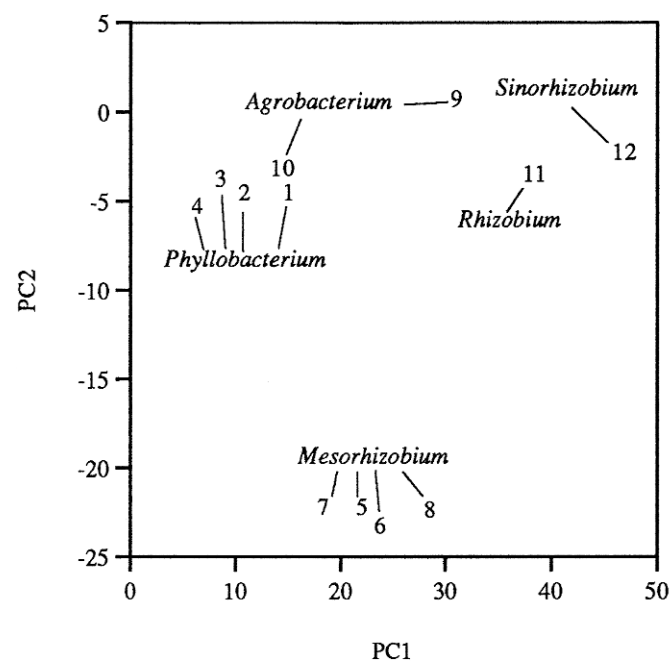


FIGURE BXII.α.141. Principal component analysis of fatty acid compositions of *Phyllobacterium* and phylogenetically related genera. Methods used are according to Jarvis et al. (1996). The following strains were investigated: *Phyllobacterium myrsinacearum* LMG 1t1^T, LMG 2t2, LMG 8225, and LMG 8229; *Mesorhizobium loti* LMG 6123, LMG 6125^T, and LMG 6126; *Mesorhizobium huakuii* LMG 14107^T, *Agrobacterium rhizogenes* LMG 150^T; *Agrobacterium tumefaciens* LMG 187^T; *Rhizobium leguminosarum* LMG 8820; and *Sinorhizobium fredii* LMG 6217^T.

Genus I. *Phyllobacterium* (ex Knösel 1962) Knösel 1984b, 356^{VP} (Effective publication: Knösel 1984a, 254)

JORIS MERGAERT AND JEAN SWINGS

Phyl.lo.bac.te'ri.um. Gr. n. *phyllos* leaf; Gr. dim. neut. n. *bakterion* a small rod; M.L. neut. n. *Phyllobacterium* leaf bacterium.

Cells cultured *in vitro* are **rod-shaped, ovoid, or reniform**. Clumping and/or **star formation** occurs when cells are grown in carrot juice medium. **Gram negative. Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Cultures are **motile** by means of polar, subpolar, or lateral flagella. Optimal temperature for growth, 28–34°C. After 1–2 days of incubation on nutrient agar, *Phyllobacterium* colonies are whitish-gray, punctiform or circular, with a colony diameter of <1 mm, and regularly edged. Most cultures are mucoid and confluent. After 4 d, colonies reach a maximum diameter of 4 mm. **Oxidase and catalase positive. Chemoorganotrophic**, using a variety of sugars or salts of organic acids as carbon sources. Do not hydrolyze starch, pectin or cellulose. **Occur in leaf nodules and the rhizosphere of higher plants.**

The mol% G + C of the DNA is: 60.3–61.3 (De Smedt and De Ley, 1977).

Type species: Phyllobacterium myrsinacearum (ex Knösel 1962) Knösel 1984b, 356 (Effective publication: Knösel 1984a, 254) (*Phyllobacterium rubiacearum* Knösel 1984a, 254.)

FURTHER DESCRIPTIVE INFORMATION

Phyllobacterium within leaf nodules of plants develop into pleomorphic cells that are rod shaped, ellipsoidal, or branched (Fig. BXII.α.142). In liquid media, especially in carrot juice medium¹, the cells appear as motile, straight rods, which form characteristic star clusters (Fig. BXII.α.143) after an initial phase of intensive swimming.

The maximum temperature at which growth occurs is 36°C; the minimum is 4–5°C. Cells heated in saline are killed within 10 min at 52°C.

Whole-cell fatty acid extracts of cells grown on modified TY medium² are composed of mainly octadecenoic acids (C_{18:1}) and cyclopropanooctadecanoic acid (C_{19:0 cyclo}), lower amounts of hydroxylated straight-chain fatty acids (C_{16:0 3OH} and C_{18:1 2OH}) and hexadecanoic acid (C_{16:0}), and minor amounts of other fatty acids.

Acid is produced from a wide range of carbohydrates without gas formation: pentoses, hexoses, maltose, inulin, glycerol, adonitol, sorbitol, and dulcitol. Starch, pectin, and cellulose are not hydrolyzed. Ammonium salts, nitrates, and most amino acids can serve as nitrogen sources. Strains may or may not reduce nitrate to nitrite.

Growth of *Phyllobacterium* is inhibited by deoxycycline, novobiocin, framycetin, and tetracycline (Lambert et al., 1990). Sensitivity towards the rhizobial peptide antibiotic trifolitoxin has been reported (Triplett et al., 1994).

Although over 400 plant species of three genera of Rubiaceae and one genus of Myrsinaceae have been reported to have bac-

terial leaf nodules (see Lersten and Horner, 1976), *Phyllobacterium* has been isolated by Knösel (1962) only from *Pavetta zimmermanniana* (Rubiaceae), *Ardisia crispa*, and *Ardisia crenata* (Myrsinaceae). Although Knösel (1984a) has stated that *Phyllobacterium* is able to induce nodules on leaves, there is still no proof for the leaf nodulation capacity of *Phyllobacterium* (Swings et al., 1992), despite more than a century of research on leaf nodulation (reviewed by Lersten and Horner, 1976).

It has been suggested that leaf-nodule bacteria produce plant growth hormones, particularly cytokinins, which are necessary for the normal function of the plant (Rodrigues-Pereira et al., 1972; Fletcher and Rhodes-Roberts, 1976). The auxin indolyl-3-acetic acid has been isolated from the growth medium of a *Phyllobacterium* strain (Lambert et al., 1990).

It has long been claimed that leaf-nodule bacteria can fix nitrogen, but this has been contested by Van Hove (1976) and Lersten and Horner (1976). The lack of the ability to fix nitrogen is also indicated by the absence of the *nif* HFK-like genes (Lam-

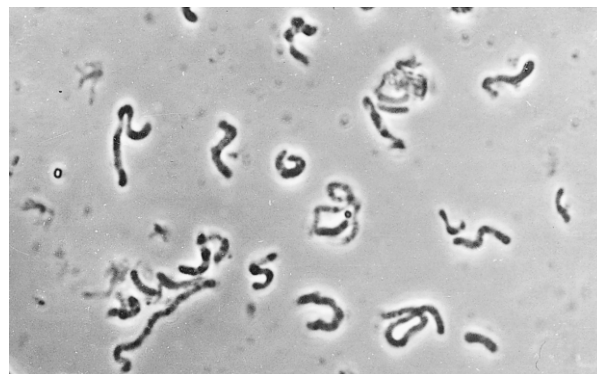


FIGURE BXII.α.142. Phase-contrast photomicrograph showing bacteroids of *Phyllobacterium myrsinacearum* (synonym *Phyllobacterium rubiacearum*) from leaf nodules of *Pavetta zimmermanniana* (× 2000).

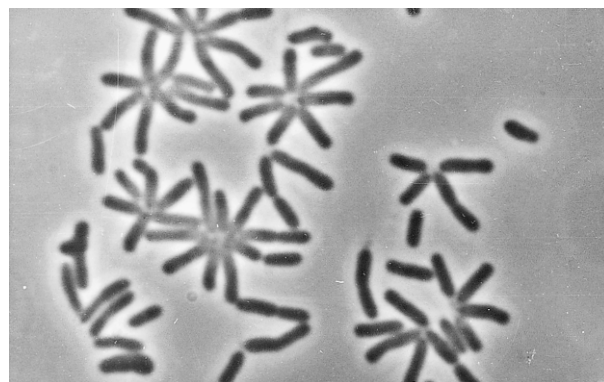


FIGURE BXII.α.143. Phase-contrast photomicrograph showing star clusters of cells of *Phyllobacterium myrsinacearum* (synonym *Phyllobacterium rubiacearum*) in carrot juice medium after incubation at 28°C for 24 h (× 2000).

1. Carrot juice medium has the following composition: fresh carrot juice, 500 ml; water, 500 ml; FeSO₄·7H₂O, 0.1 g; MnSO₄·H₂O, 0.1 g; pH 7.2. The medium is sterilized by fractional sterilization. For a solid medium, add 15.0 g/l agar. (Knösel, 1984a).

2. According to the methods described by Jarvis et al. (1996) and Sasser (1990a).

bert et al., 1990), which occur in all the classical nitrogen-fixing bacteria.

Phyllobacterium is able to interact with plant tissues, as demonstrated by the tumor induction on *Kalanchoe* plants by a Ti plasmid-carrying *Phyllobacterium* strain (Lambert et al., 1990). Tumor induction involves the attachment of bacterial cell wall sites to plant cell wall sites prior to the induction of T-DNA transfer. The chromosomal genes *chvA*, *chvB*, *exoC*, and *att* are the only ones found to be involved in attachment, but these cannot be detected in *Phyllobacterium* strains, suggesting that other genes with similar functions are present. There are no data indicating that *Phyllobacterium* is pathogenic or deleterious to plants.

A number of *Phyllobacterium* isolates show antifungal and antibacterial activities (Lambert et al., 1990). The fact that *Phyllobacterium* is a predominant bacterium on the root surface of sugar beet plant tissues, its capacity to communicate with plant tissues, and its nonpathogenic status make this bacterium an interesting new candidate for use in plant-growth promotion or biological control of soil-borne diseases. Indeed, some of the sugar beet isolates exert broad antibacterial and antifungal activity (Lambert et al., 1990). It has also been shown that a *Phyllobacterium* strain is able to reduce the symptoms of vascular *Fusarium* wilt when artificially introduced in seedlings of cotton plants prior to inoculation with *Fusarium oxysporum* f. sp. *vasinfectum* (Chen et al., 1995a).

ENRICHMENT AND ISOLATION PROCEDURES

To isolate strains from leaf nodules, Knösel (1984a) used the following procedure. Washed leaf pieces showing nodules are macerated by rubbing and placed in saline. After shaking, dilutions are plated onto carrot juice agar containing yeast extract. After incubation at 28°C, typical colonies are transferred into liquid carrot juice medium for micromorphological characterization. The cultures should be observed by phase-contrast microscopy after 24–48 h to confirm the presence of star clusters (Fig. BXII.α.143).

Lambert et al. (1990) described a procedure for isolating strains from the rhizosphere of sugar beets. The entire root system of the plant is carefully washed to remove adhering soil, then vigorously shaken for 15 minutes using a flask shaker in a phosphate-buffered saline solution containing 0.025% Tween 20. Serial dilutions of the resulting suspensions are plated on 10% trypticase soy agar. After 2 d incubation at 28°C, *Phyllobacterium* colonies are beige-colored, ~5 mm large, convex with entire margins, and very mucoid and glistening.

To extract vascular sap from cotton plants for the subsequent isolation of *Phyllobacterium*, the use of the Scholander pressure bomb has been shown to be a successful technique (Hallmann et al., 1997).

MAINTENANCE PROCEDURES

Stock cultures on trypticase soy agar or nutrient agar may be maintained at 4°C for 2–3 months. Long-term preservation can be achieved by lyophilization or by storing cell suspensions in 25% glycerol at –70°C (Swings et al., 1992).

TABLE BXII.α.127. Differential characteristics of *Phyllobacterium* and *Ochrobactrum*^{a,b}

Characteristic	<i>Phyllobacterium</i>	<i>Ochrobactrum</i>
Utilization of: ^c		
L-citruline, glutarate, meso-erythritol, glycine	–	+
L-tryptophan	+	–
Hydrolysis of:		
<i>p</i> -nitrophenyl-α-maltoside and <i>p</i> -nitrophenyl-α-xylopyranoside ^d	+	–

^aFor symbols, see standard definitions.

^bData from Swings et al. (1992).

^cUsing API 50CH, 50AO and 50AA auxanography strips (Biomérieux).

^dUsing API ZYM enzymic strips (Biomérieux).

DIFFERENTIATION OF THE GENUS *PHYLLOBACTERIUM* FROM OTHER GENERA

The genus *Phyllobacterium* is phenotypically most similar to the genus *Ochrobactrum* (Swings et al., 1992). *Phyllobacterium* is isolated from plants or the rhizosphere, whereas *Ochrobactrum* is typically of clinical origin. Table BXII.α.127 shows useful characteristics for the differentiation of the two genera from each other.

TAXONOMIC COMMENTS

The genus *Phyllobacterium* was originally restricted to bacteria that develop within leaf nodules of the tropical ornamental plants. Two species, *Phyllobacterium myrsinacearum* (from *Ardisia* leaf nodules) and *Phyllobacterium rubiacearum* (from *Pavetta* leaf nodules), have been defined on the basis of plant source, nitrate reduction, and flagellar characteristics. However, conflicting results have been obtained with regard to the nitrate-reduction capability by Lambert et al. (1990). It has become evident that the natural habitat of these bacteria is not limited to leaf nodules, and that these microorganisms are also very common on the rhizoplane and phylloplane of other plants (including sugar beet roots and *Polymnia* leaves) and in roots of cotton plants (Lambert et al., 1990; Hashidoko et al., 1994; Chen et al., 1995a; Hallmann et al., 1997). The phyllobacteria from *Ardisia*, *Pavetta*, and sugar beet roots are phenotypically very similar (Lambert et al., 1990) and the 16S rRNA sequences of the type strains of *P. myrsinacearum* and *P. rubiacearum* differ from each other by only 2 nucleotides (Yanagi and Yamasato, 1993). The sugar beet isolates show whole-cell protein-electrophoretic patterns almost identical to those of the type strain of *P. rubiacearum* and very similar to those of *P. myrsinacearum* strains (Lambert et al., 1990). Strains from these three different habitats also show very similar fatty acid compositions (see Fig. BXII.α.141 in *Phyllobacteriaceae*). DNA pairing data are not available to draw definite conclusions about speciation within the genus. Based upon their phenotypic and chemotaxonomic resemblance and for practical reasons of identification, all the phyllobacteria are united here within a single species, *Phyllobacterium myrsinacearum* (subjective synonym *Phyllobacterium rubiacearum*) (Mergaert et al., 2002).

ACKNOWLEDGMENTS

The authors are indebted to the Bijzonder Onderzoeksfonds (Belgium) for personnel grants.

List of species of the genus *Phyllobacterium*

1. ***Phyllobacterium myrsinacearum*** (ex Knösel 1962) Knösel 1984b, 356 (Effective publication: Knösel 1984a, 254) (*Phyllobacterium rubiacearum* Knösel 1984a, 254.)

myr.si.na.ce.a'rum. M.L. fem. pl. n. *Myrsinaceae* family of plants; M.L. fem. gen. pl. n. *myrsinacearum* of the myrsine family.

Cells cultured *in vitro* have a maximum size of $1.1 \times 2.2 \mu\text{m}$. The bacteroids in leaf nodules are rod-shaped or ellipsoidal, with some branched forms. A pellicle is formed in liquid media. Other characteristics are listed in Table BXII.α.128. Found in leaf nodules of tropical ornamental plants (species of Myrsinaceae and Rubiaceae) (see Figs. BXII.α.144 and BXII.α.145), on the surface of sugar beet (*Beta vulgaris*) roots (Lambert et al., 1990), in internal tissues of cotton plants (*Gossypium*) (Chen et al., 1995a; Hallmann et al., 1997), and from damaged leaf surfaces of *Polygonum sonchifolia* (Hashidoko et al., 1994).



FIGURE BXII.α.144. Photograph showing a leaf of *Ardisia crispa* with nodules located at the leaf margin.

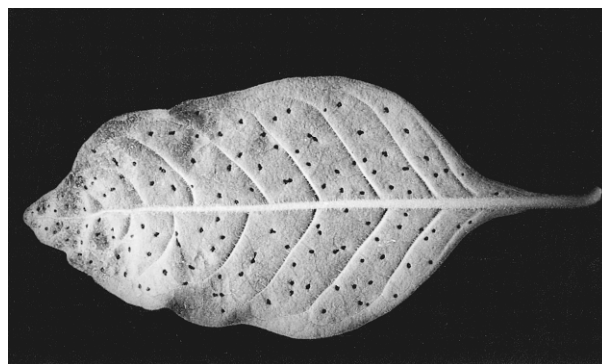


FIGURE BXII.α.145. Photograph showing a leaf of *Pavetta zimmermanniana* with nodules distributed all over the leaf blade.

TABLE BXII.α.128. Physiological and biochemical features of *Phyllobacterium myrsinacearum*^{a,b}

Characteristic	Reaction
Glucose fermentation, Voges-Proskauer, methyl red reaction, 3-ketolactose test, reducing compounds from gluconate	—
Oxidase, catalase	+
Arginine dihydrolase; arginine, ornithine, and lysine decarboxylases; urease	—
H ₂ S production from thiosulfate	+
Indole formation from tryptophan	—
Pectate breakdown, gelatin liquefaction, starch hydrolysis, Tween 20 and Tween 40 hydrolysis, deoxyribonuclease	—
Growth at pH 4.2	—
Growth at pH 5.3 and pH 7.8	+
Growth at 36°C	+
Growth in the presence of 3% NaCl	±
Growth in the presence of 10% glucose	+
Growth in litmus milk	—
Growth on NH ₄ Cl, KNO ₃ , or sodium glutamate as sole source of nitrogen	+
Utilization of the following carbohydrates as carbon source (API 50CH): ^c	
Glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, meso-inositol, D-mannitol, sorbitol, methyl-α-D-glucoside, N-acetylglucosamine, D-cellobiose, maltose, sucrose, trehalose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, D-gluconate, 2-ketogluconate	+
Utilization of the following organic acids (sodium salts) as carbon source (API 50AO): ^c	
Acetate, propionate, butyrate, isobutyrate, n-valerate, isovalerate, succinate, fumarate, glycolate, D,L-lactate, D,L-glycerate, D,L-3-hydroxybutyrate, D-malate, L-malate, pyruvate, aconitate, citrate, and p-hydroxybenzoate	+
Utilization of the following amino acids and amines as carbon source (API 50AA): ^c	
L-α-Alanine, L-leucine, L-isoleucine, L-serine, L-tryptophan, trigonelline, L-threonine, L-aspartate, L-glutamate, L-proline, betaine, D,L-4-aminobutyrate, ethanolamine, and glucosamine	+
Growth inhibition by deoxycycline (30 μg), novobiocin (30 μg), framycetin (100 μg), and tetracycline (30 μg)	+

^aSymbols: +, all strains positive; —, all strains negative; ±, all strains weakly positive.

^bData from Lambert et al. (1990).

^cUsing the API 50CH, 50AO, and 50AA auxanography strips (Biomérieux).

The mol% G + C of the DNA is: 60.3–61.3 (*T_m*) (De Smedt and De Ley, 1977).

Type strain: ATCC 43590, DSM 5892, IAM 13584, LMG 2t2 (holotype).

GenBank accession number (16S rRNA): Strain LMG 2t2, D12789.

Additional Remarks: Reference strains are LMG 1t1 (subjective synonym *P. rubiacearum*) and LMG 8225 (from sugar beet roots). The GenBank accession number (16S rRNA) for LMG 1t1 is D12790.

Genus II. Aminobacter Urakami, Araki, Oyanagi, Suzuki and Komagata 1992, 90^{VP}

TEIZI URAKAMI

Am.i.no.bac' ter. M.L. n. *aminum* amine; M.L. n. *bacter* rod, staff; M.L. masc. n. *Aminobacter* amine rod.

Rods, $0.5\text{--}0.9 \times 1.0\text{--}3.0 \mu\text{m}$, with rounded ends. Occur singly (rarely in pairs). Motile by means of subpolar flagella. Nonspore-forming. Gram negative. **Reproduction occurs by budding.** Poly- β -hydroxybutyrate granules are accumulated in the cells. Colonies are white to light yellow. Aerobic, having a strictly respiratory type of metabolism. Cells grow abundantly in nutrient broth and PYG broth. No water-soluble fluorescent pigment is produced. Methyl red and Voges-Proskauer negative. Indole and H_2S are not produced. Gelatin and starch are not hydrolyzed. **Ammonia is produced.** Denitrification does not occur. Litmus milk is not changed. Acids are produced from sugars oxidatively but not fermentatively. Monomethylamine, trimethylamine, trimethylamine-*N*-oxide, and sugars are utilized as carbon sources. Methanol, methane, and hydrogen are not utilized. Growth factors are not required. Ammonia, nitrate, urea, peptone, and methylamine are utilized as nitrogen sources. Oxidase and catalase positive. Urease negative. Good growth occurs between pH 6.0 and 8.0. No growth above pH 9.0 or below pH 5.0. Good growth occurs at 30 and 37°C; no growth at 42°C. No growth in the presence of 3% NaCl. The cellular fatty acids include a large amount of straight-chain, unsaturated $\text{C}_{18:1}$ acid. The hydroxy acids include a large amount of $\text{C}_{12:0} \text{3OH}$. The ubiquinone system is ubiquinone Q-10.

The mol% G + C of the DNA is: 62–64.

Type species: **Aminobacter aminovorans** (den Dooren de Jong 1926) Urakami, Araki, Oyanagi, Suzuki and Komagata 1992, 90 (*Pseudomonas aminovorans* den Dooren de Jong 1926, 161.)

ENRICHMENT AND ISOLATION PROCEDURES

Aminobacter strains are isolated from soils at 30°C by the enrichment culture technique, using methylamine compounds or methylformamide compounds (Urakami et al., 1990a, c). Monomethylamine, dimethylamine, trimethylamine, trimethylamine-*N*-oxide, and tetramethylammonium (TMAH) are used as methylamine compounds and *N*-methylformamide and *N,N*-dimethylformamide (DMF) are used as methylformamide compounds.

MAINTENANCE PROCEDURES

Working stock cultures should be transferred every 7 d to ensure viability. This requirement for frequent transfer appears to be independent of the type of maintenance medium or temperature of storage. Stock strains may be preserved indefinitely by lyophilization in 10% skim milk. Strains may also be preserved for at least 5 years by storage at -70°C in glycerol-PYG broth (PYG broth supplemented with 15% glycerol). Organisms are cultivated approximately 24 h in PYG broth medium, and glycerol is added to culture broth (final glycerol concentration, 15%). 1.0 ml of dense suspension of organisms is transferred to an autoclaved half-dram vial and stored in a -70°C freezer.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Acid production from carbohydrates Hugh and Leifson oxidation-fermentation basal medium (Difco) is used with each of the carbohydrates. The carbohydrates (5 g/l) are added aseptically to the autoclaved, cooled basal medium from a 10% stock solution that has been sterilized by filtration.

The GasPak[®] anaerobic system is used for anaerobic conditions. Acidification of the media is investigated up to 4 weeks.

Urease, oxidase, and catalase test Urease activity is determined on Christensen medium incubated for 7 d. The oxidase test is performed with cytochrome oxidase test paper (Nissui Seiyaku, Tokyo, Japan). Catalase activity is detected by the production of bubbles upon addition of a 3% hydrogen peroxide solution to cultures grown on agar slants of PYG medium, which consists of 0.5% peptone, 0.5% yeast extract, and 0.5% glucose (pH 7.0).

Utilization of carbon compounds Utilization of carbon compounds is carried out at a concentration of 0.15% (w/v). Utilization of methane is tested under an atmosphere containing $\text{CH}_4/\text{O}_2/\text{CO}_2$ (5:4:1) with a rotary shaker in a tightly stopped conical flask containing carbon compound-free medium. Utilization of hydrogen is tested in the same manner, but in an atmosphere containing $\text{H}_2/\text{O}_2/\text{CO}_2$ (8:1:1).

DIFFERENTIATION OF THE GENUS *AMINOBACTER* FROM OTHER GENERA

The minimal characteristics for differentiating the genus *Aminobacter* from related genera are shown in Table BXII.α.129.

Aminobacter strains resemble *Blastobacter aggregatus* and *Blastobacter denitrificans* in motility and colony pigmentation, but are differentiated from *B. aggregatus* on the basis of acid production from mannitol under aerobic conditions, utilization of ethanol and succinic acid, tolerance of NaCl, and DNA base composition, and from *B. denitrificans* on the basis of acid production from mannitol under aerobic conditions, utilization of methanol, ethanol, and sucrose, tolerance of NaCl, and denitrification (Hirsch and Muller, 1985; Urakami et al., 1992).

Aminobacter strains resemble *Hyphomicrobium* strains in that they are nonpigmented, Gram-negative, methylamine-utilizing bacteria that metabolize C_1 compounds via the serine pathway (Bellion and Hersh, 1972; Wagner and Lecvitch, 1975). However, *Aminobacter* strains are distinguished from *Hyphomicrobium* species based on formation of hyphae, utilization of methanol and carbohydrates, quinone system, and hydroxy acid composition (Hirsch, 1984; Urakami and Komagata, 1986b, 1987a, b).

TAXONOMIC COMMENTS

In his classical work, den Dooren de Jong (1926) isolated from soil enrichments with various amines (methylamine, trimethylamine, tetramethylammonium (TMAH), ethylamine, and ethylurea) a group of organisms, among which seven strains were placed in the genus *Pseudomonas*. den Dooren de Jong created the name *Pseudomonas aminovorans* for the group because of the ability of the strains to utilize various amines as sole carbon and energy sources. *Pseudomonas aminovorans*, which is a typical species of methylamine-utilizing bacteria, was included in Section V in *Bergey's Manual of Systematic Bacteriology* (Palleroni, 1984) with other *Pseudomonas* species whose natural relationships were unknown. These methylamine-utilizing bacteria were distinguished clearly from genus *Pseudomonas sensu stricto*, i.e., from the *Pseudomonas fluorescens* rRNA branch (De Vos et al., 1989), by the following characteristics: motility by subpolar flagella, multipli-

TABLE BXII.α.129. Differential characteristics of the genus *Aminobacter* and related organisms ^a

Characteristic	<i>Aminobacter</i>	<i>Blastobacter aggregatus</i>	<i>Blastobacter denitrificans</i>	<i>Brevundimonas diminuta</i>	<i>Brevundimonas vesicularis</i>	<i>Hyphomicrobium</i>	<i>Rhizobium</i>	<i>Sphingomonas</i>
<i>Colony color:</i>								
Colorless	+	+	+	+	+	+	+	—
Yellow	—	—	—	—	—	—	—	+
Colonies have viscosity	—	—	—	—	—	—	+	—
Hyphae formed	—	—	—	—	—	+	—	—
Denitrification	—	—	+	—	—	D	nd	nd
Growth with 3% NaCl	—	+	+	—	—	—	—	—
Acid from mannitol (aerobic)	+	—	—	nd	nd	—	nd	—
<i>Carbon sources:</i>								
Methanol	—	—	+	—	—	+	—	—
Monomethylamine	+	nd	nd	—	—	+	—	—
Ethanol	—	+	+	D	+	D	nd	nd
Sucrose	+	+	—	—	—	—	nd	D
Succinic acid	+	—	+	D	+	—	nd	nd
<i>Ubiquinone system:</i>								
Q-9	—	nd	nd	—	—	+	nd	—
Q-10	+	nd	nd	+	+	—	nd	+
<i>Major hydroxy acids:</i>								
C _{12:0} 3OH	+	nd	nd	+	+	—	—	—
C _{14:0} 3OH, C _{16:0}	—	nd	nd	—	—	+	—	—
C _{14:0} 3OH	—	nd	nd	—	—	—	+	+
Mol% G + C of DNA	62–64	60	65	66	66	59–65	59–64	64–66

^aFor symbols, see standard definitions; nd, no data.

cation by budding (Green and Gillis, 1989), utilization of methylamine, quinone system, cellular fatty acid composition, hydroxy fatty acid composition, DNA–DNA hybridization, and rRNA–DNA hybridization (De Ley et al., 1987; De Vos et al., 1989).

De Ley et al. (1987) studied the rRNA cistron similarities of *Pseudomonas* and *Pseudomonas*-like strains and reported that the type strain of *Pseudomonas aminovorans* was actually a member of the *Rhizobium loti* rRNA branch, not of the genus *Pseudomonas*. Furthermore, Green and Gillis (1989) pointed out the phenotypic resemblance between *Pseudomonas aminovorans* strains and the genus *Blastobacter*, which contains heterotrophic, rod-shaped, budding bacteria that do not fix nitrogen.

In 1990, the tetramethylammonium (THAH)-utilizing strain TH-3 of Urakami et al. (1990a) and the *N,N*-dimethylformamide (DMF)-utilizing strain DM-81 of Urakami et al. (1990c) were isolated and found to resemble *P. aminovorans*. Urakami et al. (1992) studied the chemotaxonomic characteristics of five methylamine-utilizing bacteria, including strains TH-3 and DM-81, and

proposed the transfer of these strains and *Pseudomonas aminovorans* to a new genus, *Aminobacter*. *Pseudomonas aminovorans* became *Aminobacter aminovorans*, and two additional species were created: *Aminobacter aganoensis* for strain TH-3^T and *Aminobacter niigataensis* for strain DM-81^T.

Although *Aminobacter* is actually a member of the *Rhizobium loti* rRNA branch as determined by rRNA–DNA hybridization (Jarvis et al., 1986; De Ley et al., 1987), it can be differentiated from *R. loti sensu stricto* on the basis of the viscosity of the colonies, utilization of methylamine, production of extracellular gum (Jordan, 1984b), hydroxy fatty acid composition (Yokota, 1989), and rRNA–DNA hybridization data (De Ley et al., 1987; De Vos et al., 1989). In addition, the failure of representative *Aminobacter* strains to reduce acetylene has been described by Green and Gillis (1989). Furthermore, these methylamine-utilizing bacteria have been isolated from soils enriched with various amines, which serve as sole carbon and energy sources.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *AMINOBACTER*

Characteristics useful in differentiation of the species of *Aminobacter* are listed in Table BXII.α.130.

TABLE BXII.α.130. Differential characteristics of the species of the genus *Aminobacter*^a

Characteristic	<i>A. aminovorans</i>	<i>A. aganoensis</i>	<i>A. niigataensis</i>
Nitrate reduction	—	w	w
Growth in peptone water	—	w or —	w or —
Oxidative production of acid from L-arabinose	+	—	—
<i>Carbon sources:</i>			
Dimethylamine	—	+	+
Formamide	—	—	w
<i>N</i> -methylformamide, <i>N,N</i> -dimethylformamide	—	—	+
Tetramethylammonium hydroxide	—	+	—

^aFor symbols, see standard definitions.

List of species of the genus *Aminobacter*

1. ***Aminobacter aminovorans*** (den Dooren de Jong 1926) Urakami, Araki, Oyanagi, Suzuki and Komagata 1992, 90^{VP} (*Pseudomonas aminovorans* den Dooren de Jong 1926, 161.) *am.i.no'vo.rans*. M.L. n. *aminum* amine; L. v. *voru* to devour; L. part. adj. *aminovorans* amine-devouring, digesting.

The characteristics are as described for the genus and as given in Tables BXII.α.129 and BXII.α.130. In addition, it has the following characteristics. Growth occurs in peptone water. Nitrate is not reduced to nitrite. Acids are produced oxidatively from L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, and glycerol. No acid production from lactose or soluble starch. Carbon sources for growth include L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, succinic acid, acetic acid, monomethylamine, trimethylamine, and trimethylamine-*N*-oxide. Lactose, soluble starch, citric acid, formic acid, ethanol, methanol, dimethylamine, formamide, *N*-methylformamide, DMF, TMAH, methane, and H₂ are not utilized.

The type strain was isolated from soil enrichments containing various amines by de Dooren de Jong in 1926.

The mol% G + C of the DNA is: 62.5 (HPLC).

Type strain: ATCC 23314, DSM 7048, JCM 7852, NCIB 9039, NCTC 10684.

GenBank accession number (16S rRNA): AJ011759.

Additional Remarks: Reference strain MS, ATCC 23819, NCIB 11591.

2. ***Aminobacter aganoensis*** Urakami, Araki, Oyanagi, Suzuki and Komagata 1992, 91^{VP} *a.ga.no.en'sis*. M.L. masc. adj. *aganoensis* coming from the Agano River, Niigata, Japan.

The characteristics are as described for the genus and as given in Tables BXII.α.129 and BXII.α.130. In addition, it has the following characteristics. Poor growth occurs in peptone water. Nitrate is weakly reduced to nitrite. Acids are produced oxidatively from D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, and glycerol. Weak acid pro-

duction from D-glucose, D-mannose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, and inositol. No acid production from L-arabinose, lactose, or soluble starch. L-Arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, succinic acid, acetic acid, monomethylamine, dimethylamine, trimethylamine, trimethylamine-*N*-oxide, and TMAH are utilized as carbon sources. Acetic acid is utilized weakly. Lactose, soluble starch, citric acid, formic acid, ethanol, methanol, formamide, *N*-methylformamide, DMF, methane, and H₂ are not utilized.

The mol% G + C of the DNA is: 63.8 (HPLC).

Type strain: Strain TH-3, DSM 7051, JCM 7854.

GenBank accession number (16S rRNA): AJ011760.

3. ***Aminobacter niigataensis*** Urakami, Araki, Oyanagi, Suzuki and Komagata 1992, 91^{VP} *ni.i.ga.ta.en'sis*. M.L. masc. adj. *niigataensis* coming from the Niigata region of Japan.

The characteristics are as described for the genus and as given in Tables BXII.α.129 and BXII.α.130. In addition, it has the following characteristics. Poor growth occurs in peptone water. Nitrate is weakly reduced to nitrite. Acid is produced oxidatively from D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, and glycerol. No acid production from L-arabinose, lactose, or soluble starch. L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, succinic acid, acetic acid, monomethylamine, dimethylamine, trimethylamine, trimethylamine-*N*-oxide, *N*-methylformamide, and DMF are utilized as carbon sources. Formamide is utilized weakly. Lactose, soluble starch, citric acid, formic acid, ethanol, methanol, TMAH, methane, and H₂ are not utilized.

The type strain was isolated as a DMF-utilizing bacterium from soil by Urakami et al. (1990c).

The mol% G + C of the DNA is: 63.2 (HPLC).

Type strain: Strain DM-81, DSM 7050, JCM 7853.

GenBank accession number (16S rRNA): AJ011761.

Genus III. *Aquamicrobium* Bambauer, Rainey, Stackebrandt and Winter 1998b, 631^{VP}
(Effective publication: Bambauer, Rainey, Stackebrandt and Winter 1998a, 300)

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A.aqua.mi.cro'bi.um. L. n. *aqua* water; L. n. *microbium* a microbe; *Aquamicrobium* a bacterium living in water/wastewater.

Gram negative, pleomorphic or regularly formed short **rods**. Motile or nonmotile. Respiratory metabolism using either O₂ or nitrate under anoxic conditions. **Nitrate reduced to nitrite or N₂**. Mesophilic. Optimal pH 6–9. Salt tolerant, up to 3% NaCl. Utilize sugars, fatty acids, and heterocyclic aromatic compounds as carbon sources. Utilize nitrate or O₂ as electron acceptors.

The mol% G + C of the DNA is: 59–65.

Type species: ***Aquamicrobium defluvii*** Bambauer, Rainey, Stackebrandt and Winter 1998b, 631 (Effective publication: Bambauer, Rainey, Stackebrandt and Winter 1998a, 300.)

ENRICHMENT AND ISOLATION PROCEDURES

Aquamicrobium defluvii strain NKK was isolated from activated sewage sludge, using thiophene-2-carboxylate as the sole carbon source and nitrate as the electron acceptor, as described in Bambauer et al. (1998a). Sludge (10% v/v) was added to mineral salts medium containing 2 mM thiophene-2-carboxylate and 10 mM nitrate. The enrichment culture was incubated at 37°C for 3 weeks, and 1 ml was transferred to fresh medium. Pure cultures were obtained after several transfers.

List of species of the genus Aquamicrobium

1. **Aquamicrobium defluvii** Bambauer, Rainey, Stackebrandt and Winter 1998a, 631^{VP} (Effective publication: Bambauer, Rainey, Stackebrandt and Winter 1998a, 300.)
de.flu'vi.i. M.L. neut. n. *defluvium* wastewater; *defluvii* from waste water.

Gram-negative rods, $0.5\text{--}0.8 \times 1.5\text{--}2.5 \mu\text{m}$. Motile. Do not form spores. Catalase and oxidase positive. Colonies are white, circular, convex, 2–4 mm in diameter after 3–5 d. Optimal temperature 30–37°C. Optimal pH 7.5–8.5. Growth

occurs at 41°C and with 2% NaCl. Vitamins required for growth. Respiratory metabolism with O₂ or nitrate as electron acceptors. Nitrate reduced to nitrite. Chemoorganotrophic, utilizing thiophene-2-carboxylate, acetate, propionate, butyrate, crotonate, glucose, fructose, mannose, xylose, mannitol, and sorbitol as carbon sources.

The mol% G + C of the DNA is: 61.7.

Type strain: NKK, CIP 105610, DSM 11603.

GenBank accession number (16S rRNA): Y15403.

Genus IV. Defluviobacter Fritsche, Auling, Andreesen and Lechner 1999b, 1325^{VP} (Effective publication: Fritsche, Auling, Andreesen and Lechner 1999a, 202)

THE EDITORIAL BOARD

De.flu.vi.bac'ter. M.L. n. *defluvium* waste water; Gr. hyp. masc. n. *bacter* rod; M.L. masc. n. *Defluviobacter* referring to its origin from activated sludge of a waste water treatment plant.

Gram-negative **rods**, occurring as single cells. Motile. **Strictly aerobic.** Oxidase and catalase positive. Does not reduce nitrate. Utilizes the following carbohydrates: D-glucose, D-fructose, D-mannose, D-ribose, D-xylose, and L-lyxose. Some amines and amino acids used as carbon sources. **Major ubiquinone is ubiquinone 10.** Major fatty acid is C_{18:1}. C_{12:0 3OH} is present in small amounts.

Spermidine is the major polyamine. The genus belongs in the *Alphaproteobacteria* and is most closely related to *Pseudaminobacter*, *Mesorhizobium*, and *Phyllobacterium*.

Type species: **Defluviobacter lusatiensis** Fritsche, Auling, Andreesen and Lechner 1999b, 1325 (Effective publication: Fritsche, Auling, Andreesen and Lechner 1999a, 202)

List of species of the genus Defluviobacter

1. **Defluviobacter lusatiensis** Fritsche, Auling, Andreesen and Lechner 1999b, 1325^{VP} (Effective publication: Fritsche, Auling, Andreesen and Lechner 1999a, 202)
lu.sa.ti.en.sis. M.L. gen. n. *lusatiae* referring to the German province of Lausitz (Latin name *Lusatia*), where the organism was isolated.

Short rods, $0.6\text{--}0.8 \times 1.5\text{--}3 \mu\text{m}$, occurring singly. Motile by means of a single polar flagellum. Do not form spores. Colonies are grayish white, circular, and mucoid, with a diameter of 2 mm after 2 d on nutrient agar. Can reach 4 mm after prolonged incubation. Optimal temperature 30–37°C (range 12–44°C). Optimal pH 7.0–7.7 (range 6.0–9.2). Grows in peptone medium. Does not form indole.

Does not hydrolyze urea, starch, gelatin, casein, DNA, Tween 80, or esculin. Utilization of carbohydrates requires yeast extract or growth factors. Carbohydrates utilized are DL- α -amino-*n*-valerate, putrescine, L-tryptophan, malonate, glutarate, and 2-ketoglutarate. Can utilize the following as carbon sources: 4-chloro-2-methylphenol, 2,4-dichlorophenol, and 4-chlorophenol. Phenol is degraded by a different set of enzymes. Does not degrade benzoate, *m*- and *p*-hydroxybenzoate, 3,4-dihydroxybenzoate, gentisate (2,5-dihydroxybenzoate), and phenylacetate. Isolated from activated sludge of an industrial waste water plant at Schwarzhede.

The mol% G + C of the DNA is: 61.4.

Type strain: S1, CIP 106844, DSM 11099.

GenBank accession number (16S rRNA): AJ132378.

Genus V. Candidatus Liberibacter Jagoueix, Bové and Garnier 1994, 385 (*Candidatus Liberobacter (sic) Jagoueix, Bové and Garnier 1994, 385*)

MONIQUE GARNIER

Li.be.ri' bac.ter. L. n. *liber* phloem; Gr. n. *bakterion* a small rod; N.L. masc. n. *Liberibacter* rod in the phloem.

Filamentous bacteria occurring in the phloem sieve tubes of plants (Fig. BXII.α.146). Round forms are also observed but have been shown to correspond to degenerating bacteria. The bacteria are also present in the hemolymph and salivary glands of insect vectors (Psyllidae) responsible for the transmission. Like most phloem-restricted bacteria, *Candidatus Liberibacter* spp. have re-

sisted *in vitro* cultivation (Garnier and Bové, 1993). The characterized species originated from sweet orange (*Citrus sinensis*). The original description is based on bacteria present in the phloem sieve tubes of sweet orange trees affected by huanglongbing (ex. greening disease) in Poona (India) and Nelspruit (South Africa).

Type species: *Candidatus Liberibacter asiaticus* Jagoueix, Bové and Garnier 1994, 385 (*Candidatus Liberobacter asiaticum* (sic) Jagoueix, Bové and Garnier 1994, 385.)

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic treatment Phylogenetic analyses of the 16S rRNA gene indicated *Candidatus Liberibacter* spp. belong to the *Alphaproteobacteria*; their closest cultivated relatives are members of the group of bacteria known as the alpha-2 subgroup belonging to the genera *Bartonella*, *Bradyrhizobium*, *Agrobacterium*, *Brucella*, and *Aflipia* (Fig. BXII.α.147) (Jagoueix et al., 1994).

Based on sequence comparisons of ribosomal protein genes of the beta operon, and on biological properties, two species have been recognized: *Candidatus Liberibacter asiaticus* and *Candidatus Liberibacter africanus*.

Strain morphology Electron microscopy measurements on thin sections show that the filamentous forms of the liberibacteria have a diameter of 0.2–0.3 μm. Variations in diameter occur between organisms and sometimes within a single organism. Round forms are larger (0.5 μm) with a less dense cytoplasm and often show plasmolysis (Figs. BXII.α.146 and BXII.α.148). The *Candidatus Liberibacter* spp. envelope is a membranous wall characteristic of Gram-negative bacteria (Fig. BXII.α.149), but the peptidoglycan layer is hardly visible (Garnier et al., 1984). There is no evidence for flagella or pili. *Candidatus L. asiaticus* and *Candidatus L. africanus* cannot be distinguished on the basis of morphology.

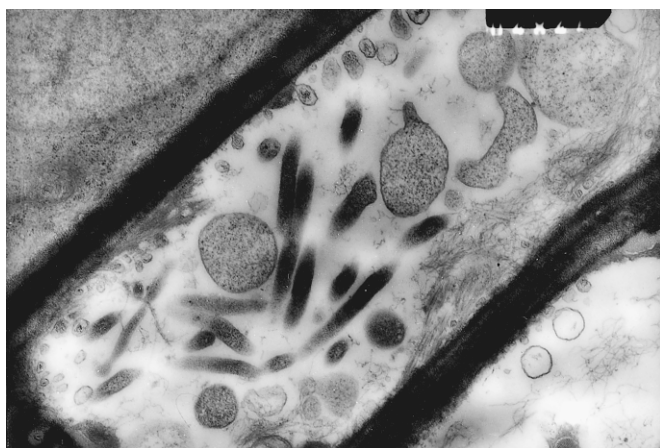


FIGURE BXII.α.146. *Candidatus Liberibacter* cells in a phloem sieve tube of huanglongbing-affected sweet orange leaves (× 12,000).

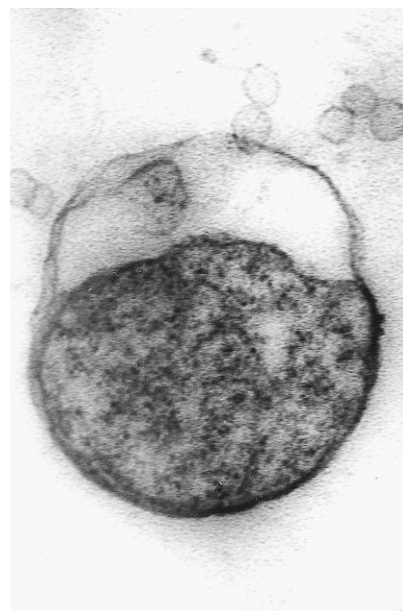


FIGURE BXII.α.148. Round plasmolyzed *Candidatus Liberibacter* (× 100,000).

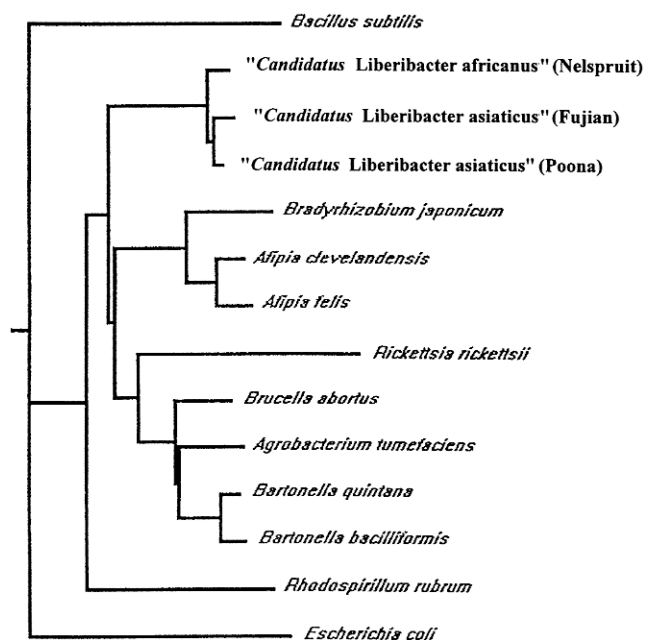


FIGURE BXII.α.147. Phylogenetic tree constructed with 16S rDNA sequences from GenBank.

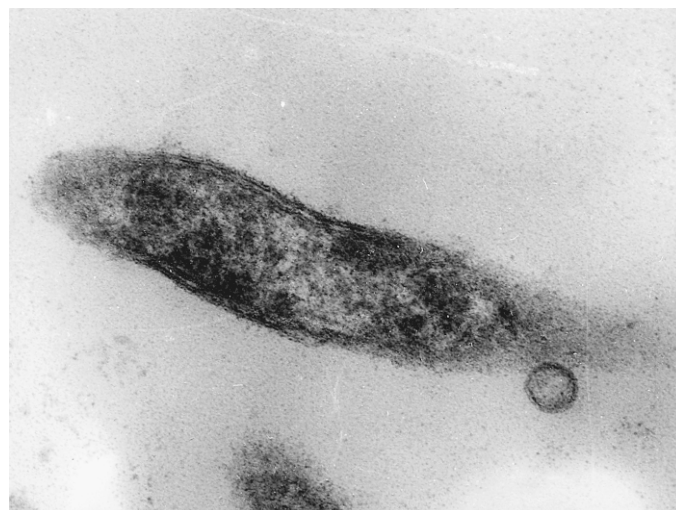


FIGURE BXII.α.149. Filamentous *Candidatus Liberibacter* cell showing the membranous cell wall (× 80,000).

Ecological data, host range *Candidatus Liberibacter* spp. are phytopathogenic bacteria affecting most, if not all, *Citrus* species and some other genera and species in the Rutaceae family. They are responsible for huanglongbing (ex. greening disease). Natural infections are limited to members of the Rutaceae plant family, but experimental transmissions by dodder (*Cuscuta* sp.) to periwinkle (*Catharanthus roseus*, Apocynaceae) and tobacco (*Nicotiana* sp., Solanaceae) have been achieved (Garnier and Bové, 1983; Bové and Garnier, 1992). *Candidatus Liberibacter asiaticus* is present in Asia (from Pakistan to China) and induces symptoms in a temperature range of 25–40°C, while *Candidatus Liberibacter africanus* is present in South and Eastern Africa as well as Cameroon and does not induce symptoms when temperatures are above 30°C (Bové et al., 1974; Garnier and Bové, 1996).

Psyllids become contaminated by feeding on infected plants; they remain infective all their life. *Candidatus Liberibacter* cells can cross the insect gut membranes and multiply into the hemolymph, from which they reach salivary glands. From there on, they can be reinoculated into plants. There is no evidence of transovarial transmission of the liberibacteria. Two psyllids, *Diaphorina citri* in Asia (Capoor et al., 1967) and *Trioza erythrae* in Africa (McLean and Oberholzer, 1965), are vectors of the liberibacteria.

Diaphorina citri has also been present in South and Central America for many years; it has recently reached Florida and the West Indies. Liberibacteria, however, are not present in the American continent and the West Indies. Recently *D. citri* has also been reported from Iran. In the Arabian Peninsula (South Western Saudi Arabia, Yemen), Mauritius, and Reunion islands, both psyllid vectors and the two *Candidatus Liberibacter* spp. are present (Garnier and Bové, 1996).

ENRICHMENT AND ISOLATION PROCEDURES

To date, all attempts to grow liberibacteria in axenic cultures have failed.

In periwinkle plants, the *Candidatus Liberibacter* cells reach higher titers than in citrus. Purified preparations of phloem tissue from infected periwinkle plants are enriched in *Candidatus Liberibacter* cells.

MAINTENANCE PROCEDURES

Strains are maintained in citrus or periwinkle plants by repeated graft-inoculations.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Specific identification Cells belonging to *Candidatus Liberibacter* can be identified by amplification and sequencing of the 16S rDNA. The complete sequence of the 16S rRNA gene is deposited in GenBank under the accession number L22532 for *Candidatus Liberibacter asiaticus* and L22533 for *Candidatus Liberibacter africanus*. Primers for specific amplification of the *Candidatus Liberibacter* 16S rDNA have been developed and yield a 1160 bp amplicon with both species (Jagoueix et al., 1996). The oligonucleotide sequences complementary to unique regions of the 16S rDNA are 5'-GCGCGTATGCAATACGAGCGGCA-3' for *Candidatus Liberibacter asiaticus* and 5'-GCGCGTATTTTATACGAGCGGCA-3' for *Candidatus Liberibacter africanus*. Digestion of the amplified DNA with *Xba*I is required for species identification.

Primers A2 (5'-TATAAAGGTTGACCTTTTCGAGTTT-3') and J5 (5'-ACAAAAGCAGAAATAGCACGAACAA-3') defined in the ribosomal protein genes *rplA* and *rplJ* of the *Candidatus Liberibacter* spp. yield amplicons of 703 bp and 669 bp for *Candidatus Liberibacter asiaticus* and *Candidatus Liberibacter africanus*, respectively (Hocquellet et al., 1999).

DNA probes specific for each species or for strains within species have also been obtained (Villechanoux et al., 1992; Hocquellet et al., 1997).

Seven serovars have been demonstrated in the *Candidatus Liberibacter* spp. with the 12 monoclonal antibodies available so far (Gao et al., 1993).

ACKNOWLEDGMENTS

Monique Garnier-Semancik died suddenly in May, 2003. She was Director of Research at INRA Bordeaux and served as director of a "Joined Research Unit" in which INRA researchers and University teachers work together. Most of her scientific contributions were to the field of phloem- and xylem-restricted plant pathogenic bacteria, which won her respect both in France and internationally.

List of species of the genus *Candidatus Liberibacter*

1. *Candidatus Liberibacter asiaticus* Jagoueix, Bové and Garnier 1994, 385 (*Candidatus Liberobacter asiaticum* (sic) Jagoueix, Bové and Garnier 1994, 385.)
as.iat' i. cus. M.L. adj. *asiaticus* from Asia.

The type strain Poona was isolated from a sweet orange tree in India.

The mol% G + C of the DNA is: not determined.

GenBank accession number (16S rRNA): AY192576, L22532.

2. *Candidatus Liberibacter africanus* Jagoueix, Bové and Garnier 1994, 379.
afr.ic' a. nus. M.L. adj. *africanus* from Africa.

The type strain Nelspruit was isolated from a sweet orange tree in South Africa.

The mol% G + C of the DNA is: not determined.

GenBank accession number (16S rRNA): L22533.

- a. *Candidatus Liberibacter africanus* subsp. *capensis* Garnier, Jagoueix-Eveillard, Cronje, le Roux and Bové 2000, 2124.

Detected in an ornamental rutaceous tree (*Calodendrum capense*) in South Africa. 16S rDNA and ribosomal protein gene sequence analysis revealed that it is different from the two previously described species but more closely related to *Candidatus L. africanus*.

The mol% G + C of the DNA is: not determined.

GenBank accession number (16S rRNA): AF137368.

Genus VI. Mesorhizobium Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 897^{VP}

WEN XIN CHEN, EN TAO WANG AND L. DAVID KUYKENDALL

Me.so.rhi.zo'bi.um. Gr. adj. *mesos* middle; M.L. neut. n. *Rhizobium* bacterial generic name; M.L. neut. n. *Mesorhizobium* the *meso*-growing rhizobium, referring to the growth rate intermediate between those of the genera *Rhizobium* and *Bradyrhizobium*.

Rods 0.4–0.9 × 1.2–3.0 µm. Commonly pleomorphic under adverse growth condition or in the root nodules as bacteroids. Usually contain granules of poly-β-hydroxybutyrate, which are refractile by phase-contrast microscopy. Nonsporeforming. Gram negative. **Motile** by one polar or subpolar flagellum or by peritrichous flagella. Aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. Optimal temperature 25–30°C. Optimal pH 6–8. Growth in yeast extract-mannitol agar (YMA)¹ produces colonies that are circular, convex, semitranslucent, raised, and mucilaginous, 2–4 mm diameter within 5–6 d for some species or 1 mm after a 7-d incubation for other species. **The generation times of *Mesorhizobium* strains range from 4–15 h.** Chemoorganotrophic, utilizing a wide range of carbohydrates and salts of organic acids as carbon sources without gas production. Cellulose and starch are not utilized. **Produces an acidic reaction in YMA.** Ammonium salts, nitrates, urea, and most amino acids are utilized as nitrogen sources. Peptone is poorly utilized. *Mesorhizobium* strains are only weakly proteolytic, but can produce a slow digestion in litmus milk; some strains form a clear serum zone. Some strains require thiamin, nicotinamide, and riboflavin for growth. **The organisms are characteristically able to invade the root hairs of a wide range of temperate, subtropical, and tropical leguminous plants, inciting production of root nodules where the bacteria reduce atmospheric nitrogen into a combined form available for the host plants.** All strains exhibit host specificity.

The mol% G + C of the DNA is: 59–64.

Type species: *Mesorhizobium loti* (Jarvis, Pankhurst, and Patel 1982) Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 898 (*Rhizobium loti* Jarvis, Pankhurst, and Patel 1982, 378.)

FURTHER DESCRIPTIVE INFORMATION

In young cultures, the cells are short rods of various cell sizes. The cell width of some strains is 0.25 µm, and the cell length of some strains is 4.0 µm (de Lajudie et al., 1998b). In old cultures, or under adverse environmental conditions, the cells are usually pleomorphic, swollen, club-shaped, or branched.

In host nodules, the bacteroids of *M. huakuii* are mostly club-shaped and approximately 40–50 times larger than the rod-shaped cells in the infection threads or *in vitro* culture. The invaded nodule cell contains many swollen bacteroids and a few rod-shaped bacteria. Microculture of bacteroids and bacteria released from bacterial nodule protoplasm reveals that only the

rod-shaped cells multiply; the club-shaped bacteroids do not (Fig. BXII.α.150). (Cao et al., 1984). The ratio of viable (may multiply) counts and total counts of bacteroids released from protoplasts of *Astragalus sinicus* is 0.27:100 for *M. huakuii*, which is between the values reported for *Sinorhizobium meliloti* in *Medicago sativa* (0.11:100) and for *Bradyrhizobium japonicum* in *Glycine max* (81.08:100) (Cao et al., 1984).

The unique phenotypic characters of this genus are the moderately slow or slow growth rate of the strains and their acid production in YMA. The generation times (4–15 h) of *Mesorhizobium* strains (Table BXII.α.131) are generally slower than those of fast growers classified in the genera *Rhizobium* and *Sinorhizobium* (<6 h) and overlap those of *Bradyrhizobium* species (>6 h). *Mesorhizobium* species produce acid in mineral medium, such as YMA, which is a characteristic of *Rhizobium* and *Sinorhizobium*; they do not produce an alkaline reaction like that characteristic of *Bradyrhizobium* (Chen et al., 1995c; Wang et al., 1998). The temperature ranges are highly strain dependent and vary between 4–42°C. Growth at 4°C and 10°C has been observed only with strains of *M. tianshanense*, and growth at 42°C is documented only for strains of *M. plurifarum*, a bacterium from tropical regions. The maximum temperature is 37–40°C for strains from temperate regions. The pH range for the genus is 4.0–10.0, except for *M. tianshanense*, which originated from an alkaline environment and can grow only in the narrow range of pH 6.0–8.0. *M. loti* is the most acid-tolerant species and grows at pH 4.0.

Mesorhizobium strains tested produce a slow digestion in litmus milk with a slightly alkaline reaction.

The bacteria in this genus fix nitrogen only in root nodules. No stem-nodule formation nor nitrogen-fixation in free-living conditions has been reported for any *Mesorhizobium* strain.

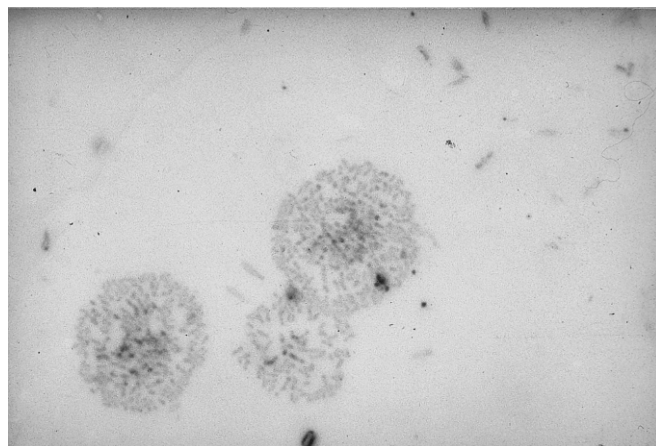


FIGURE BXII.α.150. Photomicrograph showing a microculture of bacteroids and bacteria released from bacteroidal host protoplast. There are 3 microcolonies in the lower left corner, which were developed from regular rods after a 3–4-d incubation in wet chamber. The club-shaped bacteroids elsewhere in the picture could not grow. (Reproduced with permission from Y.Z. Cao et al., Scientia Sinica (Series B) 27: 593–600, 1984, ©China Science Press.)

1. Yeast extract-mannitol agar (YMA): mannitol, 10.0 g; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; NaCl, 0.1g; CaCO₃, 4.0 g; yeast water, 100 ml (or yeast extract [Difco], 0.4 g); agar, 15.0 g; and distilled water, 1 liter; pH 6.8–7.0. Sterilize at 121°C for 15 min. The CaCO₃ is omitted in the preparation of pour plates or of liquid medium (YMB) used for turbidimetric measurement of growth. The yeast water is prepared by mixing 100 g of baker's compressed yeast with 1 liter of cold water, allowing the mixture to stand at room temperature for 1–2 h, and then steaming for 40–60 min. After the mixture has been centrifuged or allowed to settle, the clear supernatant fluid is autoclaved at 121°C for 15 min (Kleczkowska et al., 1968).

TABLE BXII.α.131. Characteristics differentiating the species of the genus *Mesorhizobium*^a

Characteristic	<i>M. loti</i> ^b	<i>M. amorphae</i> ^c	<i>M. ciceri</i> ^d	<i>M. huakuii</i> ^e	<i>M. mediterraneum</i> ^d	<i>M. plurifarum</i> ^f	<i>M. tianshanense</i> ^e
Monotrichous flagellation	+	nd	nd	+	nd	nd	d
Colony diameter, mm (incubation time)	1 (7 d)	1 (7 d)	2–4 (3–5 d)	2–4 (5–6 d)	2 (5–7 d)	0.5–2 (2–3 d)	1–2 (5–7 d)
Generation time (h)	nd	5–13	<6	4–6	>5	nd	5–15
Maximum growth temperature (°C)	39	37	40	37	40	42	37
Maximum NaCl tolerated for growth (% w/v)	2.0	<1.0	2.0	1.0	2.0	1.0	1.0
pH range for growth	4.0–10.0	5.0–9.0	5.0–10.0	5.0–9.5	9.5	nd	6.0–8.0
<i>Sole carbon sources:</i>							
D- and L-Arabinose/maltose	+	nd	+	+	+	+	d
D-Fructose/sucrose	+	+	+	+	+	+	d
D- and L-Fucose/L-proline	+	nd	+	–	+	+	d
Fumarate	+	nd	+	+	+	+	–
Inositol	+	+	+	–	+	+	–
D- and L-Malate	+	+	+	+	+	+	–
D-Raffinose	d	+	–	+	–	d	d
Erythritol	d	nd	–	–	–	–	+
L-Aspartate	–	nd	+	–	–	d	d
L-Ornithine	d	+	+	+	–	nd	+
L-Xylose	+	nd	+	+	–	+	+
β-Alanine	d	+	+	–	–	nd	d
Mol% G + C of DNA (<i>T_m</i>)	59–64	64	63–64	59–64	63–64	63–64	59–63

^aFor symbols, see standard definitions.^bData from Jarvis et al. (1982).^cData from Wang et al. (1999b).^dData from Nour et al. (1994a, 1995).^eData from Chen et al. (1991b, 1995c).^fData from de Lajudie et al. (1994, 1998b).

Strains contain 1–5 plasmids, with molecular sizes ranging from 60–940 kb, depending on the species and strain. Symbiotic plasmids carrying the *nod* and *nif* genes have been reported in *M. huakuii* (Chan et al., 1988; Xu and Murooka, 1995; Zou et al., 1997) and *M. amorphae* (Wang et al., 1999b). In other species, the symbiotic genes might be carried on the chromosome, as in *Bradyrhizobium*, since no symbiosis-controlling plasmids have been identified (Wang et al., 1998). Novel and complex chromosomal arrangements have been reported for the nodulation genes of *M. loti* (Scott et al., 1996). The gene *nodB* is separated from *nodACIJ* in *M. loti*, but they are in the same operon in other legume-nodulating bacteria. Lateral transfer of symbiotic genes in the field has been reported between *M. loti* and other related bacteria (Sullivan et al., 1995), and a transferable chromosomal element carrying the symbiotic genes has been identified for *M. loti* (Sullivan and Ronson, 1998).

M. loti strains produce extracellular polysaccharides, which contain uronic acid, galactose, and glucose (Jarvis et al., 1982). Somatic antigens are highly strain specific, but internal antigens show a reaction of identity among strains and can be used to distinguish *M. loti* from other *Rhizobium* species (Jarvis et al., 1982). No serological crossreactions (immunofluorescence) have been observed among chickpea rhizobia and other rhizobia (Kingsley and Bohlool, 1983). As analyzed by the method of Vincent (1982), antisera to strains of *M. huakuii* either do not react or do so only weakly with strains of *Astragalus hamosus* and *A. adsurgens* (Chan et al., 1988).

Most strains are resistant to streptomycin, erythromycin, ampicillin, kanamycin, polymyxin, and nystatin at concentrations of 10–30 µg/ml (Chan et al., 1988; Chen et al., 1991b, 1995c; Wang et al., 1998). Two species isolated from chickpea plants, *M. mediterraneum* and *M. ciceri* (Nour et al., 1994a, 1995), can be differentiated by antibiotic tests: in contrast to *M. ciceri*, *M. mediterraneum* is susceptible to carbenicillin and resistant to chloram-

phenicol, nalidixic acid, and trimethoprim-sulfamethoxazole (Nour et al., 1995).

Strains of *Mesorhizobium* occur in vast areas from arctic to tropic regions, according to their hosts. The host range of the species within genus *Mesorhizobium* varies from a single legume species to several species in different genera. Some *Mesorhizobium* species share hosts with each other or with bacteria from other rhizobial genera. Four restriction-fragment-length-polymorphism (RFLP) groups of *Cicer*-nodulating bacteria have been identified by Kuykendall et al. (1993a). *Cicer arietinum* is a host for *M. ciceri*, *M. mediterraneum*, and two other genomic species (Nour et al., 1994a, 1995). *Acacia* species have been reported to nodulate with *M. plurifarum*, *Sinorhizobium teranga*, and some as yet unnamed species (de Lajudie et al., 1994). Among *Mesorhizobium* species, *M. tianshanense* has the broadest host range.

ENRICHMENT AND ISOLATION PROCEDURES

Isolation from root nodules and soil is by the techniques described for the genus *Rhizobium*.

MAINTENANCE PROCEDURES

A suitable medium is YMA. Storage recommendations are the same as given for the genus *Rhizobium*.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Procedures for testing special characters are the same as given for the genus *Rhizobium*.

DIFFERENTIATION OF THE GENUS *MESORHIZOBIUM* FROM OTHER GENERA

Phenotypically, the moderately slow growth rate and acid production in YMA can differentiate *Mesorhizobium* strains from other related bacteria, such as *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Allorhizobium*, *Bradyrhizobium*, and *Azorhizobium*.

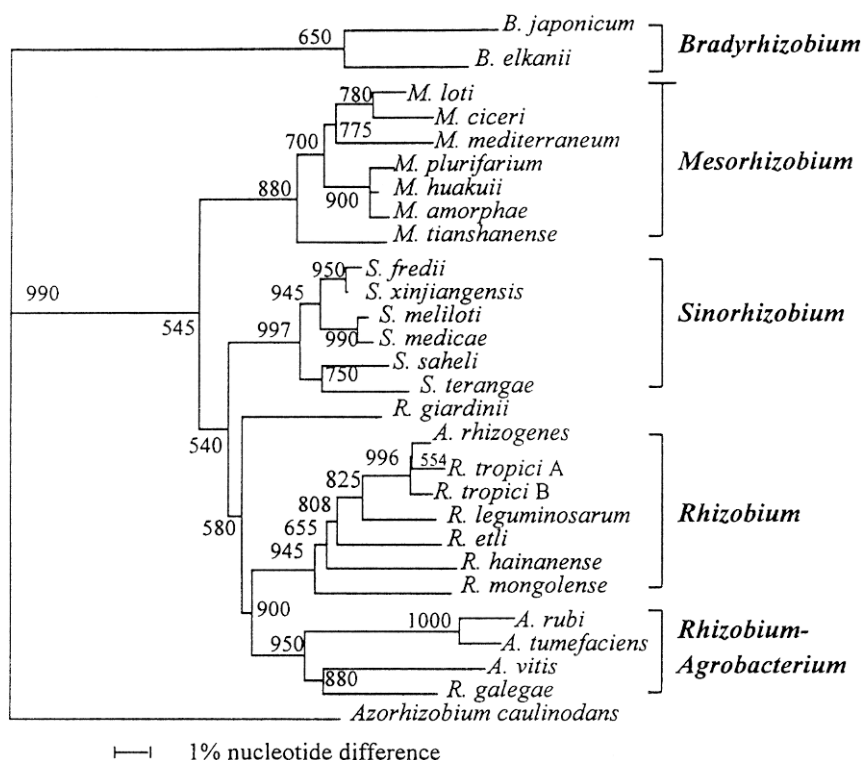


FIGURE BXII.α.151. Dendrogram showing the phylogenetic relationships of species of *Mesorhizobium* and related genera. 16S rRNA gene sequences were obtained from GenBank, DDBJ, and EMBL (analyzed by Z.Y. Tan). The similarity values were calculated with Jukes-Cantor coefficient and the neighbor-joining method was used for construction of the phylogenetic tree and 1000 subsamples were generated for bootstrap analysis using the package of PHYLIP version 3.572c (Felsenstein, 1993).

Analyses of rRNA sequences can differentiate *Mesorhizobium* species from other related bacteria on the phylogenetic basis (Fig. BXII.α.151). Ribotyping or PCR-based RFLP of 16S rRNA genes can serve as a simple and quick method for identification of *Mesorhizobium* strains (Laguerre et al., 1994; Wang et al., 1998). *Mesorhizobium* species have fingerprinting patterns different from other related bacteria and form a group distinct from other bacteria according to cluster analysis of RFLP patterns.

Other differences among the genus *Mesorhizobium* and related bacteria have been demonstrated by numerical taxonomy, SDS-PAGE patterns of total cell proteins, MLEE patterns, PCR-based fingerprinting (ribotyping, ARDRA, RAPD, AFLP), and total DNA-DNA relatedness.

TAXONOMIC COMMENTS

The genus *Mesorhizobium*, together with some other genera, such as *Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium*, constitutes an rRNA cluster in the *Alphaproteobacteria* class. The 16S rRNA sequence similarities between *M. loti* (type species of genus *Mesorhizobium*) and representatives of related genera, specifically *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, *Sinorhizobium fredii*, *Bradyrhizobium japonicum*, and *Azorhizobium caulinodans*, are 93.2, 93.1, 95.1, 87.7, and 89.4, respectively.

In the first edition of *Bergey's Manual of Systematic Bacteriology*, all of the legume root nodule bacteria were divided into two genera: *Rhizobium* and *Bradyrhizobium* (Jordan, 1984a). *M. loti*, the type species of the genus *Mesorhizobium*, was included in the genus *Rhizobium* as *R. loti* (Jarvis et al., 1982). Several new species phylogenetically related to *R. loti* were later described, including

R. huakuii from *Astragalus sinicus* (Chen et al., 1991b), *R. ciceri*, and *R. mediterraneum* from *Cicer arietinum* (Nour et al., 1994a, 1995), and *R. tianshanense* from several leguminous plants (Chen et al., 1995c). It was recognized that *R. loti* and its relatives were quite different from both the fast-growing symbiotic species, such as *R. leguminosarum* and *S. meliloti*, and the slow growing bacteria in the genus *Bradyrhizobium*, now in a new family, the *Bradyrhizobiaceae*. Comparative analyses of the complete 16S rRNA sequences from representative strains for the species indicated that all these species clustered in a close branch distinct from related bacteria (Nour et al., 1995; Tan et al., 1997; de Lajudie et al., 1998b). The strains of *Mesorhizobium* produce acid in YMA, and they generally exhibit growth rates slower than those of *R. leguminosarum* and *S. meliloti*. Consequently, there is good agreement that these organisms warrant the designation of a new genus in the family *Rhizobiaceae* (Lindström et al., 1995; Young and Haukka, 1996). The name *Mesorhizobium*, referring to the moderately slow growth rate, was first suggested by W.X. Chen and coworkers and afterwards validly proposed (Jarvis et al., 1997) to include five species: *M. ciceri*, *M. huakuii*, *M. loti*, *M. mediterraneum*, and *M. tianshanense*. Subsequently, three new species have been described: *M. plurifarum*, from the tropical trees *Acacia senegal*, *Leucaena leucocephala*, and *Prosopis juliflora* (de Lajudie et al., 1998b); *M. amorphae*, from *Amorpha fruticosa*, originating from Chinese soils (Wang et al., 1999b); and *M. chacoense* (Velázquez et al., 2001).

Although 16S rRNA gene analysis can differentiate *Mesorhizobium* from other related genera, it is not as useful for defining the species within this genus. For example, *M. loti* is not coherent

and consists of strains with differing phylogenies, since there are more differences among the strains within this species than there are among different *Mesorhizobium* species (Laguerre et al., 1997). Thus, the definition of species within this genus is based mainly upon DNA–DNA hybridization, associated with distinctive features.

Diversity has been investigated for some *Mesorhizobium* species using different methods. Two genomic groups have been distinguished within *M. loti* by DNA–DNA hybridization and by 16S rRNA analyses (de Lajudie et al., 1998b). *M. plurifarum* has been reported to contain two subgroups divided by protein SDS-PAGE and DNA–DNA hybridization (de Lajudie et al., 1998b). *M. amorphae* has been reported as one of the three *Mesorhizobium* groups from *A. fruticosa*, each of which contains strains with different enzyme electrophoretic types (ET) and different plasmid con-

tents. These three groups have different phylogenies within the *Mesorhizobium* branch but share the same symbiotic plasmid (Wang et al., 1998). *M. huakuii* has been described for nine strains isolated from *A. sinicus*, grown in the southern part of China. Recently 200 strains from the same host species growing in seven provinces of China have been divided into four genotypic groups (including *M. huakuii*) by PCR-RFLP of 16S rRNA analysis (Zhong, personal communication). Further taxonomic work would be desirable to clarify the relationships among the defined species and new or unnamed groups.

ACKNOWLEDGMENTS

The authors are indebted to Dr. M. Gillis, Dr. E. Martínez-Romero, Dr. K. Lindström, and other members in the International Subcommittee for the Taxonomy of *Rhizobium*/*Agrobacterium* for their valuable advice.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *MESORHIZOBIUM*

The differential characteristics of the species of *Mesorhizobium* are indicated in Table BXII.α.131.

List of species of the genus *Mesorhizobium*

1. ***Mesorhizobium loti*** (Jarvis Pankhurst, and Patel 1982) Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 898^{VP} (*Rhizobium loti* Jarvis Pankhurst, and Patel 1982, 378.)

loti. M.L. masc. n. *Lotus* generic name of leguminous plants; M.L. gen. n. *loti* of *Lotus*.

The characteristics are as described for the genus and as indicated in Table BXII.α.131. Cells are motile, predominantly by one polar or subpolar flagellum. Bacteroids in nodules are club-shaped and branched and contain inclusion bodies composed of poly-β-hydroxybutyrate (Jarvis et al., 1982). Strains usually lack plasmids, but a plasmid has recently been found in strain NZP 2213 (Wang et al., 1998). All strains utilize one or more of the following carbohydrates as sole carbon sources and form acidic products from them: glucose, galactose, fructose, arabinose, xylose, rhamnose, maltose, sucrose, lactose, trehalose, raffinose, mannitol, and dulcitol (Jarvis et al., 1982).

Strains normally form nitrogen-fixing root nodules on the following hosts: *Lotus corniculatus*, *Lotus tenuis*, *Lotus japonicus*, *Lotus krylovii*, *Lotus filicalius*, and *Lotus schoelleri*. Ineffective nodules are formed on *L. pedunculatus*, *L. huspidus*, and *L. angustissimus*. Information concerning additional plant hosts is presented by Jarvis et al. (1982).

The mol% G + C of the DNA is: 59–64 (T_m).

Type strain: ATCC 33669, DSM 2626, LMG 6125, NZP 2213.

GenBank accession number (16S rRNA): D14514, X67229.

2. ***Mesorhizobium amorphae*** Wang, van Berkum, Sui, Beyene, Chen and Martínez-Romero 1999b, 63^{VP} *amorphae*. M.L. gen. n. *amorphae* of *Amorpha*, a genus of plant with which the species forms a nitrogen-fixing symbiosis.

The characteristics are as described for the genus and as indicated in Table BXII.α.131. The cell size is 0.41–0.65 × 0.47–1.68 μm. Produces acid in mineral-salt medium containing mannitol and alkaline in litmus milk. The generation times are from 6–13 h in YM broth. The species can use L-arabinose, D-fructose, D-glucose, rhamnose, sucrose,

L-xylose, inositol, fumarate, alanine, and ornithine as sole carbon sources and ammonium salts and tyrosine as sole nitrogen sources. Strains are resistant to cefoperazone (75 μg/ml) and tetracycline (30 μg/ml). Usually, strains harbor one to three plasmids (150–930 kb). The genes for symbiosis reside on the 930-kb plasmids. There is only one *nifH* gene copy. Strains of this species have been isolated from nodules of *Amorpha fruticosa* growing in north China. No nodulation by the reference strain, ATCC 19665, has been observed on any selected host plant of other described microsymbiont species, except its original one, and *Amorpha fruticosa* does not nodulate with any type strains of other described species within the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Azorhizobium* (Wang et al., 1999b).

The species can be differentiated at the molecular level from other *Mesorhizobium* species and related genera by PCR-RFLP of the 16S rRNA gene, multilocus enzyme electrophoresis (MLEE) patterns, DNA–DNA relatedness, and 16S rRNA sequence.

The DNA–DNA similarity between *M. amorphae* and *M. loti* is 35.0% (Southern blotting method), and sequence similarity of 16S rRNA gene between them is 98.2%.

The mol% G + C of the DNA is: 63–64 (T_m).

Type strain: ATCC 19665.

GenBank accession number (16S rRNA): AF041442.

3. ***Mesorhizobium chacoense*** Velázquez, Igual, Willems, Fernández, Muñoz, Mateos, Abril, Toro, Normand, Cervantes, Gillis and Martínez-Molina 2001, 1019^{VP} *chacoense*. N.L. neut. adj. *chacoense* from El Chaco, Argentina, where the type strain was isolated.

Motile Gram-negative rods. Colonies on yeast mannitol agar opaque, white, and convex. Aerobic. Acid produced from adonitol, L-arabinose, galactose, lactose, maltose, melibiose, rhamnose, sucrose, and trehalose in NH₄NO₃-containing media. N-acetylglucosaminidases, α-maltosidases, and β-xylosidases produced. Resistant to ciprofloxacin, cloxacillin, and erythromycin. Isolated from trees of the genus *Prosopis*. Form root nodules and fix nitrogen in association with *P. alba*, *P. chilensis*, and *P. flexuosa*.

The mol% G + C of the DNA is: 62 (HPLC).

Type strain: LMG 19008.

GenBank accession number (16S rRNA): AJ278249.

4. **Mesorhizobium ciceri** (Nour, Fernandez, Normand and Cleyet-Marel 1994a) Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 897^{VP} (*Rhizobium ciceri* Nour, Fernandez, Normand and Cleyet-Marel 1994a, 520.) *ci' ce.ri*. L. gen. n. *ciceri* of *Cicer*.

The characteristics are as described for the genus and as indicated in Table BXII.α.131. Sole carbon sources are glycerol, arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol, N-acetylglucosamine, maltose, saccharose, trehalose, D-turanose, L-fucose, D-arabitol, succinate, fumarate, malate, pyruvate, L-histidine, tryptophan, L-glutamate, D,L-kynurenine, L-proline, betaine, D,L-4-aminobutyrate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, glucosamine, galactose, D,L-lactate, L-tyrosine, D-lyxose, glycerate, L-aspartate, L-ornithine, β-alanine, L-xylose, L-alanine, and L-leucine (Nour et al., 1994b). No serological crossreaction is observed between this organism and 55 strains of *R. leguminosarum*, *B. japonicum*, and others (Kingsley and Bohlool, 1983). Strains of this species are isolated from chickpeas grown in uninoculated fields over a wide geographic range, including Spain, the United States of America, India, Russia, Turkey, Morocco, and Syria. Of 71 chickpea isolates tested, 70 nodulate only the original host plant and do not nodulate the 88 species belonging to Fabaceae and Mimosaceae (Gaur and Sen, 1979). Consequently, this species may be considered a monospecific cross-nodulation system.

This species can be distinguished at the molecular level from other *Mesorhizobium* species and related genera by DNA–DNA relatedness, PCR-RFLP of 16S rRNA, and 16S rRNA sequencing.

The DNA similarity between *M. ciceri* and *M. loti* is 7.5% (*S*₁ nuclease method). The 16S rRNA sequence similarity between them is 99.8%.

The mol% G + C of the DNA is: 63–64 (*T*_m).

Type strain: UPM-Ca7, ATCC 51585, DSM 11540, LMG 14898.

GenBank accession number (16S rRNA): U07934.

5. **Mesorhizobium huakuii** (Chen, Li, Qi, Wang, Yuan and Li 1991b) Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel, and Gillis 1997, 897^{VP} (*Rhizobium huakuii* Chen, Li, Qi, Wang, Yuan and Li 1991b, 278.) *hua.kui'i*. N.L. gen. n. *huakuii* in honor of Huakui Chen, a Chinese professor of soil microbiology, who is the pioneer in investigating the microsymbionts isolated from *Astragalus sinicus*.

The characteristics are as described for the genus and as indicated in Table BXII.α.131. Motile by means of a single polar or subpolar flagellum. Cells usually contain granules of poly-β-hydroxybutyrate. The bacteroids in nodules are mostly club-shaped, in vast amounts and approximately 40–50 times larger than the unswollen rod-shaped bacterium (Cao et al., 1984). The generation time of the strains in the species varies from 4–6 h. Strains use D-arabinose, cellobiose, D-fructose, D-glucose, lactose, sucrose, maltose, D-raffinose, L(+) -rhamnose, malate, fumarate, oxalate, or succinate as a sole carbon source. Ammonium nitrate, urea,

and many amino acids, including arginine, citrulline, lysine, methionine, ornithine, threonine, tryptophan, and tyrosine, are utilized as sole nitrogen sources. DL-Leucine and valine are not utilized. Some strains require thiamin, nicotinamide, and riboflavin for growth (Chen et al., 1991b). Strains are resistant to the following antibiotics (μg/ml): streptomycin, 10; tetracycline, 10; erythromycin, 20; penicillin, 10; kanamycin, 5; polymyxin, 10; nystatin, 30; neomycin, 10. Most strains contain 1–5 plasmids (66–942 kb). Usually, the *nod* and *nif* genes are carried on the largest plasmid (942 kb) as a gene cluster (Xu and Murooka, 1995; Zou et al., 1997). The other plasmids are also related to symbiosis. A derivative strain cured of the middle-sized plasmid does not nodulate the host plant, has an altered lipopolysaccharide, and grows more slowly than the parent strain. Curing the strain of the smallest plasmid results in delayed nodulation and loss of nitrogen-fixation ability. Curing the strain of each of these plasmids reduces the acid tolerance (Zou et al., 1997). The original host of the species is *Astragalus sinicus*, a green manure crop grown in rice fields in the southern parts of China, Japan, and Korea. This species either cannot nodulate other leguminosarum species or can do so only weakly, and isolates from nodules of 24 species of 8 genera of Leguminosae cannot nodulate *A. sinicus*, except for isolates from *Desmodium hoterotyllum* (Chen and Shu, 1944). Consequently, the *M. huakuii* symbiosis may be a monospecific cross-nodulation system (Chen et al., 1992).

This species can be differentiated at the molecular level from other *Mesorhizobium* species and related genera by SDS-PAGE of whole-cell proteins, DNA–DNA relatedness, and 16S rRNA sequence.

The DNA–DNA similarity between type strains of *M. loti* and *M. huakuii* is 22.3%. The 16S rRNA sequence similarity between them is 98.3%.

The mol% G + C of the DNA is: 59–64 (*T*_m).

Type strain: 103, ATCC 51122, CCBAU 2609, DSM 6573, IAM 14158.

GenBank accession number (16S rRNA): D12797.

6. **Mesorhizobium mediterraneum** (Nour, Normand, Cleyet-Marel and Fernandez 1995) Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 898^{VP} (*Rhizobium mediterraneum* Nour, Normand, Cleyet-Marel and Fernandez 1995, 647.) *me.di.ter.ra' ne.um*. L. adj. *mediterraneum* midland, inland.

Characteristics are as described for the genus and as indicated in Table BXII.α.131. Strains that have been tested can grow at 4°C and cannot grow at pH 5.0 and 10.0; these differentiate them from *M. ciceri*. Sole carbon sources include 39 compounds that are the same as those used by *M. ciceri*. D-Lyxose, DL-glycerate, L-aspartate, L-ornithine, β-alanine, L-xylose, L-alanine, and L-leucine are not used (Nour et al., 1994b). Strains comprise a single serological group (Kingsley and Bohlool, 1983). This species may be a monospecific cross-nodulation system (Gaur and Sen, 1979). It can be distinguished at the molecular level from other *Mesorhizobium* species and related genera by DNA–DNA relatedness, PCR-RFLP of the intergenic spacer (IGS) between the 16S and 23S rRNA genes (the average length of the IGS DNA is 1000 bp), and total 16S rRNA sequence analysis.

The DNA–DNA similarity between *M. mediterraneum* and

M. loti is 31%, and the 16S rRNA sequence similarity between them is 97.2%.

The mol% G + C of the DNA is: 63–64 (T_m).

Type strain: ATCC 51670, UPM-Ca36, DSM 11555.

GenBank accession number (16S rRNA): L38825.

7. **Mesorhizobium plurifarum** de Lajudie, Willems, Nick, Moreira, Molouba, Hoste, Torck, Neyra, Collins, Lindström, Dreyfus and Gillis 1998b, 380^{VP}
plu.ri.fa'ri.um. M.L. adj. *plurifarum* from adv. *plurifarum* in a different place, referring to the fact that this species contains strains isolated from several places in East Africa, West Africa, and South America.

The characteristics are described as for the genus and as indicated in Table BXII.α.131. Cells are motile in liquid media. Colonies on YMA are beige, round, convex to drop-like, 0.5–2.0 mm in diameter within 2–3 d at 30°C. Strains utilize glycerol, arabinose, ribose, L-xylose, L-sorbose, D-mannose, rhamnose, inositol, mannitol, sorbitol, N-acetylglucosamine, D-cellobiose, maltose, lactose, D-melibiose, D-raffinose, xylitol, D-turanose, D-lyxose, succinate, fumarate, glycolate, D,L-lactate, D-malate, L-malate, pyruvate, L-histidine, L-glutamate, L-ornithine, L-arginine, L-proline, betaine, and glucosamine as sole carbon sources. Erythritol, methyl-L-xyloside, amygdalin, glutarate, D-tartrate, D-mandelate, L-mandelate, glycine, D-(–)-alanine, L-norleucine, D,L-norvaline, L-serine, L-cysteine, L-citrulline, creatine, diamminobutane, and spermine are utilized (de Lajudie et al., 1994).

Most of the strains can nodulate *Acacia senegal*, *A. tortilis*, *A. nilotica*, *A. seyal*, *Leucaena leucocephala*, and *Neptunia oleracea*, but not *Sesbania rostrata*, *S. pubescens*, *S. grandiflora*, *Ononis repens*, and *Lotus corniculatus* (Lortet et al., 1996; de Lajudie et al., 1998b). This species can be differentiated at the molecular level from other *Mesorhizobium* species and related genera by SDS-PAGE of whole-cell proteins, DNA–DNA hybridization, and 16S rRNA sequence analysis.

The DNA–DNA similarity between this species and *M. loti* is 10–13%. The 16S rRNA sequence similarity between this species and *M. loti* is 98.0%.

The mol% G + C of the DNA is: 62.6–64.4 (T_m).

Type strain: LMG 11892, ORS 1032.

GenBank accession number (16S rRNA): Y14158.

8. **Mesorhizobium tianshanense** Jarvis, van Berkum, Chen, Nour, Fernandez, Cleyet-Marel and Gillis 1997, 898^{VP} (*Rhizobium tianshanense* Chen, Wang, Wang, Li, Chen and Li 1995c, 158.)

tian.shan.en'se. M.L. adj. *tianshanense* referring to the Tianshan Mountains in the Xingjiang region of the People's Republic of China, where strains were isolated.

The characteristics are described as for the genus and as indicated in Table BXII.α.131. Cells of some strains move by means of peritrichous flagella, others by means of polar and subpolar flagella. The generation time of strains varies from 5–15 h in YM broth. Even the strains with a slow growth rate (generation time >8 h) produce an acid reaction in YMA. All tested strains use D-glucose, mannitol, glycerol, D-xylose, D-fucose, erythritol, D,L-proline, L-(+)-glutamic acid, and rhamnose; some strains can use lactose, maltose, sucrose, pyruvate, raffinose, sorbitol, fructose, mannose, arabinose, citrate, and dulcitol. Tagatose, galactose, melibiose, sorbose, inulin, benzoate, malate, vanillic acid, inositol, salicytol, fumarate, pyrocatechol, starch, and dextrin are not utilized. Sole nitrogen sources are ammonium salts, nitrate, and many kinds of amino acids, including glutamate, glycyl-L-leucine, valine, threonine, arginine, proline, and glycine. No megaplasmid has been detected, but some strains harbor three plasmids (166–468 kb) (Zou and Chen, unpublished). Strains have been isolated from *Glycyrrhiza pallidiflora*, *G. uralensis*, *Glycine max*, *Sophora alopecuroides*, *Swainsonia salsula*, *Caragana polourensis*, and *Halimodendron holodendron* growing in Xinjiang Region of China. Most of these plants are wild and indigenous to that region, except *Glycine max*, a cultivated crop that originated in northeastern China. The type strain A-1BS nodulates all of the original host species of *M. tianshanense* strains but not *Pisum sativum*, *Medicago sativa*, *Phaseolus vulgaris*, *Trifolium repens*, *Lotus corniculatus*, *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Vigna unguiculata*, and *Astragalus sinicus*. Therefore, the hosts of *M. tianshanense* form a single cross-nodulation group (Chen et al., 1995c).

The species can be differentiated from other *Mesorhizobium* species and related genera by phenotypic features, DNA–DNA relatedness, and 16S rRNA sequence.

The DNA–DNA similarity between the type strain of this species and that of *M. loti* is 4.4% (Southern blotting method), and the 16S rRNA gene sequence similarity between them is 97.6%.

The mol% G + C of the DNA is: 59–63 (T_m).

Type strain: A-1BS, CCAU 3306, DSM 11417.

GenBank accession number (16S rRNA): AF041447, U71079.

Other Organisms

Four unnamed species within the genus *Mesorhizobium* have been reported for some symbiotic or nonsymbiotic strains that were isolated from the rhizosphere of *Lotus corniculatus* (Sullivan et al., 1995, 1996). These isolates have been identified by RFLP of different genes from *M. loti*, including exopolysaccharide genes and symbiotic genes, total DNA–DNA hybridization, MLEE, and 16S rRNA gene sequencing. They are considered to be potential recipients that can acquire and express symbiotic-controlling genes from related microsymbiotic strains.

Two other *Mesorhizobium* groups were also identified when the species *M. amorphae* was described (Wang et al., 1999b). These two groups were also isolated from *Amorpha fruticosa*, and they share moderate DNA–DNA similarity (35–50%) and the same symbiotic plasmid with *M. amorphae* but have distinct phylogenies of their 16S rRNA genes. They also have high phenotypic similarities to *M. amorphae*. These might be included in *M. amorphae* when further evidence is obtained.

Genus VII. Pseudaminobacter Kämpfer, Müller, Mau, Neef, Auling, Busse, Osborn and Stolz 1999, 894^{VP}

PETER KÄMPFER

Pseud.ami.no.bac'ter. Gr. adj. *pseudos* false; M.L. *aminobacter* generic name of a bacterium; *Pseudaminobacter* false aminobacters.

Rods 0.5–0.8 × 1.0–2.0 µm, with rounded ends. **Motile**. Gram negative. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Oxidase and catalase positive**. Colonies on nutrient agar at 25°C are circular, entire, slightly convex and smooth, glistening, and pale beige. Contains ubiquinone Q-10. Major polyamines are spermidine, *sym*-homospermidine, and putrescine. Polar lipid pattern characterized by nearly equal amounts of phosphatidylcholine, phosphatidylglycerol, phosphatidyltrimethylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, and diphosphatidylglycerol. The major cellular fatty acid is C_{18:1}. The hydroxy acids include the hydroxylated fatty acid C_{15:0 iso 3OH}. Isolated from wastewater and river water.

The mol% G + C of the DNA is: 62.9–63.9.

Type species: Pseudaminobacter salicylatoxidans Kämpfer, Müller, Mau, Neef, Auling, Busse, Osborn and Stolz 1999, 894.

FURTHER DESCRIPTIVE INFORMATION

P. salicylatoxidans BN12^T was described as an aerobic bacterium, able to degrade substituted naphthalenesulfonates and substituted salicylates and originated from a 6-aminonaphthalene-2-sulfonate degrading microbial consortium from the German river Elbe (Nörtemann, 1987). *P. defluvi* THI 051^T was isolated during a study on thiobacilli (Katayama-Fujimura et al., 1983,

1984b), but it became soon obvious that the organism was not an authentic *Thiobacillus* species.

Growth occurs on nutrient agar (Oxoid), tryptone soya agar (Oxoid), trypticase soy broth (BBL) supplemented with 1.5% (w/v) agar (BBL), and R2A agar (Oxoid).

DIFFERENTIATION OF THE GENUS *PSEUDAMINOBACTER* FROM OTHER GENERA

Table BXII.α.132 lists the main differential characteristics of the genera belonging to this rRNA group.

TAXONOMIC COMMENTS

16S rDNA sequence analyses of the type strains of the two *Pseudaminobacter* species clearly place the genus in the family within the *Alphaproteobacteria*. In phylogenetic trees, the highest similarities occur with *Aminobacter* species (>96.7%). Together with the genera *Deffluvibacter*, *Mesorhizobium*, *Phyllobacterium*, *Sinorhizobium*, *Ochrobactrum*, and *Aminobacter* (sequence similarities between 16S rRNA genes are above 93%), they form a branch referred to as the group of bacteria known as the alpha-2-subgroup of the *Proteobacteria*. In Fig. BXII.α.152 a phylogenetic tree is presented.

At present, each species is represented by only one strain (Kämpfer et al., 1999).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *PSEUDAMINOBACTER*

The two species can be differentiated based on several biochemical tests as indicated in Table BXII.α.133.

TABLE BXII.α.132. Features that differentiate the genus *Pseudaminobacter* from related genera of the *Alphaproteobacteria*^{a,b}

Characteristic	<i>Pseudaminobacter</i>	<i>Aminobacter</i> ^c	<i>Deffluvibacter</i>	<i>Mesorhizobium</i>	<i>Ochrobactrum</i>	<i>Phyllobacterium</i>
<i>Major polyamines:</i>						
Putrescine	+	+		+		+
Spermidine	+		+		+	
<i>sym</i> -Homospermidine	+	+		+		+
Presence of fatty acid C _{12:0 3OH}	–	+	+	+	–	–
<i>Assimilation of:</i>						
Malonate			+	–	–	–
Glutarate	D	D	+	–	+	–
Norvaline			+	–	+	–
L-Tryptophan	–	–	+	D	–	D
Putrescine	–	–	+	–	–	–
Nitrate reduction			–	–	+	+

^aSymbols: see standard definitions; blank space, not determined.

^bData from Fritsche et al. (1999a) and Kämpfer et al. (1999).

^cThe genus *Chelatobacter* is not listed separately, because it is considered as a later synonym of *Aminobacter* (Kämpfer et al., 2002)

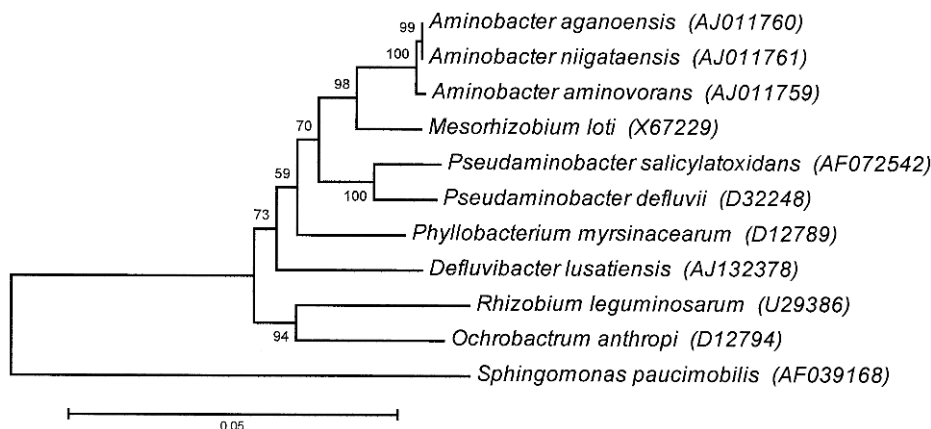


FIGURE BXII.α.152. Phylogenetic tree based on 16S rRNA sequences of *Pseudaminobacter* species and close relatives. The underlying distance matrix has been calculated with the neighbor joining tool of the ARB package using the Jukes-Cantor correction. All gaps in the sequences were excluded. *S. terrae* was used as the outgroup. Accession numbers of the analyzed sequences are indicated. Bar = 10% sequence divergence.

List of species of the genus *Pseudaminobacter*

- Pseudaminobacter salicylatoxidans*** Kämpfer, Müller, Mau, Neef, Auling, Busse, Osborn and Stolz 1999, 894^{VP}
sa.li.cy.lat.oxi'dans. N.L. *salicylatoxidans* oxidizing salicylate, because the organism oxidizes salicylate in an unusual manner.

The characteristics are as described for the genus and as listed in Tables BXII.α.132 and BXII.α.133, with the following additional features. Cell size, 0.5–0.8 × 1.0–1.5 μm. Colonies on nutrient agar are circular, entire, slightly convex and smooth, glistening, and beige, 1–3 mm in diameter. Growth occurs at 20–40°C. The major polyamines are spermidine, *sym*-homospermidine, and putrescine. Utilize adonitol, *cis*-aconitate, D-maltose, D-trehalose, glutarate, and L-malate but not L-arabinose, D-cellobiose, D-galactose, D-mannose, sucrose, L-ornithine, and L-serine. Do not hydrolyze *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl-β-D-glucopyranoside. Isolated from a microbial consortium originating from the river Elbe, Germany.

The mol% G + C of the DNA is: 63.9 (HPLC).

Type strain: BN12, CIP 106963, DSM 6986.

GenBank accession number (16S rRNA): AF072542.

- Pseudaminobacter defluvii*** Kämpfer, Müller, Mau, Neef, Auling, Busse, Osborn and Stolz 1999, 895^{VP}
de.flu'vi.i. L. neut. gen. n. *fluvius* wastewater, because the organism was isolated from activated sludge.

The characteristics are as described for the genus and as listed in Tables BXII.α.132 and BXII.α.133, with the following additional features. Coccoid to rod-shaped cells, 0.5–0.8 × 0.8–1.2 μm. Colonies on nutrient agar are circular, entire, slightly convex and smooth, glistening, and beige, 1–3 mm in diameter. Growth occurs between 10° and 40°C. No growth occurs at 5° or 45°C. Utilize D-glucose, ribose, D-xylose, acetate, and propionate but not D-maltose, D-trehalose, adonitol, mannitol, sorbitol, L-malate, and L-aspartate. Isolated from a Japanese activated sludge enriched with thiocyanate.

The mol% G + C of the DNA is: 62.9 (HPLC).

Type strain: THI 051, CIP 107185, IFO 14570.

GenBank accession number (16S rRNA): D32248.

TABLE BXII.α.133. Differential characteristics of the type strains of *Pseudaminobacter* species^a

Characteristic ^b	<i>P. salicylatoxidans</i> strain BN12	<i>P. defluvii</i> strain THI 051
<i>Acid produced from:</i> ^c		
D-Mannitol	w	—
Dulcitol	w	—
Melibiose	w	—
<i>Utilization of:</i> ^d		
D-Maltose	+	—
D-Trehalose	+	—
Adonitol	+	—
D-Mannitol	+	—
D-Sorbitol	+	—
<i>cis</i> -Aconitate	+	—
Glutarate	+	—
L-Malate	+	—
L-Aspartate	+	—
L-Ornithine	—	+
L-Serine	—	+
4-Hydroxybenzoate	+	—

^aSymbols: +, positive; —, negative; w, weakly positive. Test results given in the table were read after 72 h of incubation at 30°C.

^bTests for hydrolysis of various substrates (not listed in this table) failed to differentiate the two strains. Both type strains hydrolyzed bis-*p*-nitrophenyl-phosphate, *p*-nitrophenyl-phosphorylcholine, and L-alanine-*p*-nitroanilide. Neither strain hydrolyzed esculin, *p*-nitrophenyl-β-L-galactopyranoside, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-phosphorylcholine, 2-deoxythymidine-5'-*p*-nitrophenyl-phosphate, and L-glutamate-γ-3-carboxy-*p*-nitroanilide.

^cAcid formation from carbohydrates in most cases was very weak (even after prolonged incubation). Both strains showed weak acid formation from D-glucose. Neither strain formed acid from lactose, sucrose, salicin, adonitol, inositol, sorbitol, L-arabinose, raffinose, rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl-D-glucoside, and erythritol.

^dBoth strains utilized N-acetyl-D-glucosamine, D-glucose, D-ribose, D-xylose, acetate, propionate, 4-aminobutyrate, DL-3-hydroxybutyrate, DL-lactate, oxoglutarate, pyruvate, L-alanine, β-alanine, L-histidine, L-leucine, and L-proline. Neither strain utilized L-arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, gluconate, α-D-melibiose, L-rhamnose, sucrose, salicin, D-inositol, maltitol, putrescine, *trans*-aconitate, adipate, azelate, citrate, fumarate, itaconate, mesaconate, suberate, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, and L-phenylacetate.

Family V. **Methylocystaceae** *fam. nov.*

JOHN P. BOWMAN

Me.thy.lo.cyst.a' ce.ae. M.L. masc. n. *Methylocystis* type genus of the family; *-aceae* ending to denote a family; M.L. fem. pl. n. *Methylocystaceae* the *Methylocystis* family.

Cells are **pyriform, vibrioid, reniform, or rod-like** in shape, sometimes arranged in rosettes. **Reproduce by budding or binary division. Produce heat- and desiccation-resistant exospores or lipoidal cysts.** Motility varies; if present, cells are propelled by a polar and/or subpolar flagellar tuft. Cells contain **type II intracytoplasmic membranes**, which are arranged as layers in parallel to the periphery of the cell wall. Possess an **aerobic, strictly respiratory metabolism. Obligately methanotrophic, utilizing only methane and methanol as sole carbon and energy sources.** C₂ and other C₁ compounds are not utilized. Fix formaldehyde for cell carbon via the **serine pathway**. Enzymes for the Benson-Calvin cycle pathway are absent. The tricarboxylic acid pathway is complete. **Fix atmospheric nitrogen** by means of an oxygen-sensitive nitrogenase. Mesophilic. Nonhalophilic. Major habitats include soils, freshwater sediments, and groundwater. Primary fatty acids are C_{18:1 ω8c} and C_{18:1 ω7c}. Primary quinone is ubiquinone-8 (Q-8).

The mol% G + C of the DNA is: 61–67 (T_m).

Type genus: **Methylocystis** (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751.

FURTHER DESCRIPTIVE INFORMATION

Circumscription of the family Bacteria that have the capability to oxidize and utilize methane as a sole carbon and energy source are referred to as methanotrophic bacteria or methanotrophs (Whittenbury et al., 1970b). The original description of the family *Methylococcaceae* (Whittenbury and Krieg, 1984) included two distinct groups of methanotrophs referred to in the literature as “type I” and “type II” methanotrophs. In addition, there is a third group called the “type X” methanotrophs (Hanson and Hanson, 1996), which represents a subgroup of type I methanotrophs. Type I and II methanotrophs possess extensive biological differences, which are morphological, biochemical, chemotaxonomic, and phylogenetic in nature. The order “*Methylococcales*”, which includes the type I methanotrophs, is classified in the *Gammaproteobacteria* and is thus dealt with in another part of this volume. The family *Methylocystaceae* represents all type II methanotrophs and includes the genera *Methylocystis* and *Methylosinus*. Phenotypic properties and fatty acid components differentiating these genera are shown in Tables BXII.α.134 and BXII.α.135, respectively. The facultative methylotroph *Methylobacterium* represents a distinct taxonomic group and does not have close enough taxonomic association with *Methylocystis* or *Methylosinus* to be included in the family *Methylocystaceae*.

Methane oxidation The major characteristic that separates methanotrophs from other procaryotes is their ability to utilize methane as a sole carbon and energy source. Methanotrophs oxidize methane to CO₂ in a dissimilatory pathway that generates energy and allows access to metabolizable carbon units in the form of formaldehyde. The first step of this pathway is the oxidation of methane to methanol by action of methane monooxygenase (MMO). Methanol is then oxidized to formaldehyde by a pyrroloquinol quinone-containing methanol dehydrogenase. Formaldehyde is then either used for cell carbon (see below) or oxidized to formate and then CO₂, these final steps forming reducing equivalents needed to drive methane oxidation.

TABLE BXII.α.134. Differentiation of the major types of methanotrophs^a

Characteristic	Type I ^b	Type II ^c	Type X ^d
<i>Resting stages:</i>			
<i>Azotobacter</i> -type cysts	+	—	+
Exospores	—	+	—
Lipoidal cysts	—	+	—
<i>Intracytoplasmic membranes:</i>			
Type I	+	—	+
Type II	—	+	—
Soluble methane monooxygenase (sMMO)	—	+	—
<i>Carbon assimilation pathway:</i>			
RuMP	+	—	+
Serine	—	+	—
Benson-Calvin cycle enzymes	—	—	+
Fatty acid carbon chain length	16	18	16
<i>Major quinone:</i>			
MQ-8	+	—	+
Q-8	+	+	—
Mol% G + C (T _m)	43–60	61–67	56–65

^aFor symbols see standard definitions.

^bGenera include *Methylosphaera*, *Methylobacter*, *Methylobacterium*, and *Methylomonas*; family *Methylococcaceae*, class *Gammaproteobacteria*.

^cGenera include *Methylosinus* and *Methylocystis*; family *Methylocystaceae*, class *Alphaproteobacteria*.

^dGenera include *Methylococcus*; family *Methylococcaceae*, class *Gammaproteobacteria*.

^eMay have no resting stage.

TABLE BXII.α.135. Phospholipid fatty acid profiles of the genera *Methylosinus* and *Methylocystis*^a

Fatty acids	Percent composition	
	<i>Methylocystis</i>	<i>Methylosinus</i>
C _{16:0}	0.9–5.0	0.7–2.2
C _{16:1 ω7c}	0.3–2.6	9.3–14.2
C _{18:1 ω13c}	0–4.9	0
C _{18:1 ω9c}	0–0.4	0–0.2
C _{18:1 ω8c}	61.0–74.6	65.9–70.5
C _{18:1 ω7c}	15.3–18.6	12.8–17.7
C _{18:0}	2.1–5.8	0–0.2
C _{18:1 cyclo}	1.0–4.7	0–2.2
C _{19:0 cyclo}	1.5–4.9	0–3.0

^aData from Bowman et al. (1993c).

In all type II methanotrophs, MMO is present in a membrane-bound form and is referred to as particulate MMO (pMMO) (Semrau et al., 1995). Synthesis of a cytoplasmic version of MMO, referred to as soluble MMO (sMMO) (Dalton, 1992; Murrell, 1992; Lipscomb, 1994), is common in type II methanotrophs but is relatively rare in type I methanotrophs (Bowman et al., 1993a; Bowman and Sayler, 1994; Miguez et al., 1997). Although pMMO and sMMO are genetically distinct (Martin and Murrell, 1995), they are controlled by a common copper-inducible regulatory pathway (Nielsen et al., 1997). Soluble MMO is copper-repressible and contains three components. The first component is a hydroxylase that contains an unusual non-heme iron-oxygen linked active site. The second component is a regulatory protein, and the third is a ferredoxin-based reductase. Particulate MMO has a high affinity to methane, allowing for more efficient growth

yields than with sMMO. sMMO appears to be an adaptation to copper-limiting growth conditions (Hanson and Hanson, 1996). However, sMMO has an exceptionally broad substrate specificity and can co-metabolize an extraordinarily wide range of aliphatic, aromatic, and heterocyclic compounds (Haber et al., 1984). This property has been exploited biotechnologically, with methanotrophs being proposed for organic transformation applications in industrial applications (Lidstrom and Stirling, 1990) and as bioremediation agents (Hanson and Hanson, 1996).

The amino acid sequences of the polypeptides and the nucleotide sequences of the genes (*mmoA*, *mmoB*, *mmoC* and *mmoX*) of sMMO of type I and II methanotrophs show a high degree of conservation (Stainthorpe et al., 1991; Murrell, 1992). These genes have been used to develop general oligonucleotide probes that are specific for different types of methanotrophs (Holmes et al., 1995b; McDonald and Murrell, 1997a, b; Miguez et al., 1997; Dedysh et al., 1998b). Several physiological comparisons have been drawn between methanotrophs and ammonia-oxidizing bacteria because both groups are capable of oxidizing methane and ammonia by similar means (Hanson and Hanson, 1996). For example, type II methanotrophs are able to oxidize ammonia to nitrite and nitrous oxide, although the oxidation rates are lower than those in ammonia-oxidizing bacteria (Yoshinari, 1985). An evolutionary link has been drawn between type I methanotrophs and ammonia-oxidizers (such as *Nitrosococcus oceanii*) owing to the similarity of pMMO (*pmoA* gene) and ammonia monooxygenase genes (Holmes et al., 1995a). However, the *pmoA* sequences from type II methanotrophs are comparatively more divergent compared to *pmoA* gene sequences from type I methanotrophs (Holmes et al., 1995a; Bodrossy et al., 1997).

C₁ carbon assimilation Type II methanotrophs are specialized to utilize C₁ compounds, including methane, methanol, and formaldehyde. C₂⁺ (acetate, D-glucose, etc.) and other C₁ compounds (formate, methylamine, etc.) are not utilized as energy sources, but they may be used as supplementary carbon sources when cells are grown in the presence of methane or methanol (Hanson et al., 1992). Formaldehyde is toxic to methanotrophic cells at relatively low concentrations and so is often not tested as a carbon source. In addition, freshly isolated methanotrophs often prove sensitive to methanol (due to accumulation of formaldehyde); consequently, methanol should be tested only at low concentrations (10–30 mM) or provided as a vapor in a sealed container. Methanotrophs can be “trained” to tolerate higher concentrations of methanol (Lidstrom, 1988). Type II methanotrophs use the serine pathway to incorporate formaldehyde into synthetic pathways (Large and Quayle, 1963), in contrast to type I methanotrophs, which utilize the ribulose monophosphate (RuMP) pathway to fix carbon (Strom et al., 1974). The serine pathway has been described in detail by Hou (1984). The enzyme α -hydroxypyruvate reductase is often used to detect the serine pathway and thus can be used as a direct way to distinguish type I and II methanotrophs; however, some type I methanotrophs may possess a low level of activity for α -hydroxypyruvate reductase. Type II methanotrophs may also be distinguished by their lack of key enzymes (i.e., hexose phosphate synthase) for the RuMP pathway (Colby et al., 1979).

Intracytoplasmic membranes When methanotrophs are grown in the presence of methane and methanol, characteristic intracytoplasmic membranes (ICMs) are formed from convolutions of the cytoplasmic membrane. ICMs are about 80–90 nm thick, appear as typical lipid bilayers, and in type II methano-

trophs are arranged in pairs parallel to the cytoplasmic membrane (type II ICM) (Best and Higgins, 1981) (Fig. BXII.153). The relative ICM content in cells is enhanced under reduced methane tensions, at elevated copper ion concentrations, and when a stable and rapid rate of methane transfer is available (Best and Higgins, 1981; Scott et al., 1981).

Ecology Methane is an important greenhouse gas and the most abundant organic gas in the atmosphere (Crutzen, 1991). Methanotrophs are the largest global methane sink and as a result are ubiquitous in nature. They produce the highest and most active populations in environments with a stable gas exchange in which both oxygen and methane are readily available (Reeburgh et al., 1993). Recent evidence suggests methanotrophs make up a high proportion of the total bacterial biomass (up to 40%) in many aquatic environments and surface sediments (Ross et al., 1997; P.I. Boon, personal communication). Stable isotopic analyses (methane is highly ¹³C-depleted) indicate that a considerable proportion of carbon found in aquatic life at different trophic levels has its origin from methanotrophic bacteria (Boschker et al., 1998; Boon et al., unpublished).

Many studies have used indirect, culture-independent means to study the ecology of type II methanotrophs. These include indirect immunofluorescence (Reed and Dugan, 1978; Smirnova and Archipova, 1981), lipid signatures (Nichols et al., 1985, 1987; Bowman et al., 1991a) and probes or PCR to detect pMMO and sMMO (Tsien and Hanson, 1992; Hanson et al., 1993; Brusseau et al., 1994; McDonald et al., 1995, 1996; McDonald and Murrell, 1997b; Ross et al., 1997; Dedysh et al., 1998b), methanol dehydrogenase (McDonald and Murrell, 1997a), and 16S rRNA genes (Brusseau et al., 1994; Holmes et al., 1995a, 1996; McDonald et al., 1996). This use of culture-independent means is perhaps due to the effort and time necessary to obtain pure cultures. In only a few instances have studies coupled culture-independent and -dependent investigations, thereby permitting a definitive identification of the type II methanotrophs present in environmental samples. In any case, type II methanotrophs are ubiquitous in

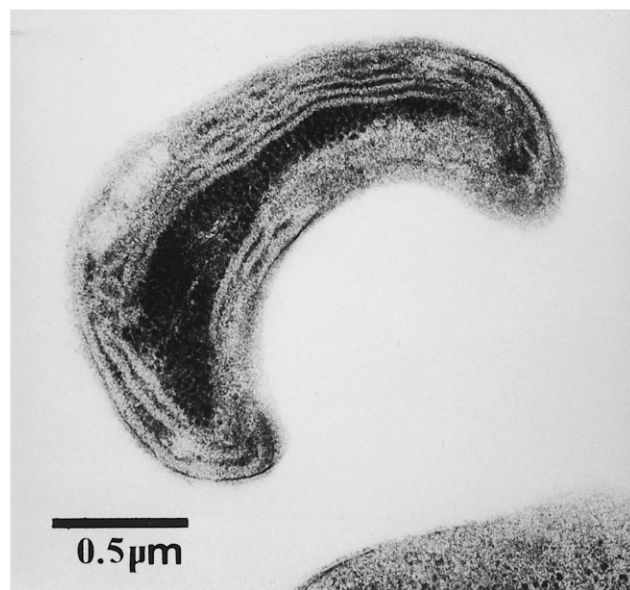


FIGURE BXII.153. Electron micrograph of a thin section of *Methylocystis parvus* showing the typical ultrastructure of type II intracytoplasmic membranes.

aquatic and terrestrial ecosystems and have been successfully isolated or enriched from marshes and swamps; roots of aquatic macrophytes; sediments of rivers and streams; rice paddies; ponds, lakes; soils of meadows, forests, peat bogs, and tundra; groundwater; and sewage sludge (Whittenbury et al., 1970b; Gal'chenko, 1977, 1994; Haubold, 1978; Heyer et al., 1984; Nichols et al., 1985; Bowman et al., 1993a, c; Hanson et al., 1993; Vecherskaya et al., 1993; Boon et al., 1996; Calhoun and King, 1998; Dedysh et al., 1998b). In general, type II methanotrophs appear to have a narrower distribution than do type I methanotrophs because they have not been isolated from marine habitats or low and high temperature ecosystems and have not been found to form endosymbioses.

Type II methanotrophs outcompete type I methanotrophs in copper- and nitrogen-limiting oligotrophic environments such as groundwater and soil (Bowman et al., 1993a; Graham et al., 1993) and in environments with low oxygen tensions where methane is not limiting for growth (Amaral et al., 1995a, b). Though as common as type I methanotrophs in various freshwater environments (Saralov et al., 1984; Gal'chenko, 1994; Ross et al., 1997), type II methanotrophs preferentially concentrate in surface layers of sediment (Reed and Dugan, 1978; Hanson et al., 1993).

Genus I. *Methylocystis* (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}

JOHN P. BOWMAN

Me.thyl.o.cys'tis. Fr. *methyle* the methyl radical; *cystis* bag; M.L. n. *Methylocystis* methyl bag.

Cells are **small, rod-like to reniform** in shape, $0.3\text{--}0.5 \times 0.5\text{--}1.5 \mu\text{m}$, usually arranged singly. **Reproduce by binary division**. Nonmotile. Cells contain **type II intracytoplasmic membranes**, which are arranged as multiple layers along the periphery of the cell wall. May form **cylindrical spinae**. Cells may contain a **desiccation-resistant lipoidal cyst resting stage**. Form inclusions of poly- β -hydroxybutyrate. **Aerobic**, possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Obligately methanotrophic**, utilizing only methane and methanol as sole carbon and energy sources. C_2+ and other C_1 compounds are not utilized. Fixes formaldehyde for cell carbon via the serine pathway. Enzymes for the Benson-Calvin cycle pathway are absent and the tricarboxylic acid pathway is complete. **Fixes atmospheric nitrogen**. Mesophilic, neutrophilic, and nonhalophilic ecophysiology with optimal growth at about $25\text{--}30^\circ\text{C}$ and pH 7.0. Major habitats include rice paddy soils, sewage, and freshwater sediments. Primary fatty acids are $\text{C}_{18:1\omega8c}$ and $\text{C}_{18:1\omega7c}$. Primary quinone is ubiquinone-8 (Q-8). Member of the family *Methylocystaceae* in the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 61–67 (T_m).

Type species: *Methylocystis parvus* (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751.

FURTHER DESCRIPTIVE INFORMATION

Methylocystis strains grow well in nitrate mineral salts (NMS) medium¹ under a methane/air headspace (usually provided in a

that have high methane fluxes. When present together, type I and II methanotrophs can produce either mutually beneficial or antagonistic effects depending on the strains compared (Starostina et al., 1995; Pashkova et al., 1997).

FURTHER READING

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1:1 ratio). Crimped sealed serum vials, anaerobic jars, or desiccators with an inlet tap provide useful vessels for cultivating strains. Colonies on NMS agar or silica gels appear initially white but become pale pink following extended incubation, probably due to accumulated cytochromes. Colonies have a convex and circular shape with an entire edge and butyrous consistency. *Methylocystis echinoides* colonies are pinpoint on standard agar nitrate mineral salts media but develop larger colonies on silica gel solidified media. Over extended incubation times, many *Methylocystis* strains form a diffusible tan pigment, the intensity of which varies from strain to strain but is thought to be linked to cyst formation (Whittenbury et al., 1970a).

Fatty acid profiles *Methylocystis* species possess a fatty acid profile consisting of mainly $\text{C}_{18:1\omega8c}$, $\text{C}_{18:1\omega7c}$, and $\text{C}_{18:0}$ with smaller amounts of cyclopropane fatty acids (Table BXII.α.135). The profile is very similar to that of *Methylosinus*, differing only in a greater abundance of $\text{C}_{18:0}$ and a lower level of $\text{C}_{16:1\omega7c}$ (Bowman et al., 1991a). The fatty acid $\text{C}_{18:1\omega8c}$ is an unusual feature that is almost unique to the Family *Methylocystaceae* and is very rarely encountered in other procaryotes (Nichols et al., 1985; Bowman et al., 1991a). In ecological studies $\text{C}_{18:1\omega8c}$ has become a very useful signature for the detection and quantification of type II methanotroph populations in environmental samples (Nichols et al., 1985, 1987; Guckert et al., 1991; Boon et al., 1996; Guezennec and Fiala-Medioni, 1996; Sundh et al., 1997). The major lipopolysaccharide-derived hydroxy fatty acids

water), 1 ml of a trace element solution (1 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.075 g H_3BO_3 , 0.25 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.25 g EDTA disodium salt, 0.1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.05 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, per liter distilled water), and (optional) 1.5% agar. The solution is sterilized by autoclaving. After cooling to approximately 50°C , 10 ml of phosphate buffer (71.6 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 26 g KH_2PO_4 per liter distilled water) is added.

1. Nitrate mineral salts (NMS) medium consists of the following: 100 ml of a $10 \times$ NMS salt stock solution (10 g KNO_3 , 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g anhydrous CaCl_2 , dissolved in 700 ml distilled water) is diluted in distilled water to approximately 1 liter. To this is added 0.1 ml of iron EDTA solution (3.8 g FeEDTA in 100 ml distilled water), 1 ml molybdate solution (0.26 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per liter distilled

include $C_{14:0\ 3OH}$ and $C_{18:0\ 3OH}$. In addition, *Methylocystis* species also possess unusual ω -1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992) that make up about 15% of the total hydroxy fatty acids. These hydroxy acids have been previously detected in freshwater sediments (Mendoza et al., 1987) and *Methylocystis* and *Methylosinus* represent the first recognized biological source of these particular lipids. *Methylocystis* species contain a suite of polar lipids that vary slightly between strains, possibly due to cultivation conditions (Andreev and Galchenko, 1983). Polar lipids present include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and lysophosphatidylglycerol. The major quinone in *Methylocystis* is Q-8 (Collins and Green, 1985).

ENRICHMENT AND ISOLATION PROCEDURES

Methylocystis species can be enriched and isolated from the micro-aerobic and aerobic sediments of freshwater lakes and rivers, soil, and sewage samples. A small amount of sample is added to a liquid NMS medium in serum vials or in cotton wool-stoppered flasks placed within airtight containers. Methane is added directly to the vials and containers, usually by first removing a portion of the headspace. The best methane/air ratio to use is equivocal but should be in a range of 1:10–1:1. The methane should be of high purity. Natural gas may contain acetylene, which is a suicide substrate of pMMO and sMMO and will hinder or prevent growth even at low concentrations. Static incubation should then proceed at 25–30°C. Growth from the enrichments can then be plated onto mineral salts agar plates which are then incubated under 1:1 methane/air at 25°C.

One of the most problematic areas of methanotroph study lies in obtaining pure cultures. In practically all situations, methanotroph enrichments are severely contaminated by nonmethanotrophic (often methylotrophic) bacteria, which can easily overgrow and/or predate the cultures. Due to the relatively slow growth of type II methanotrophs such as *Methylocystis* and the humid conditions in which plates are incubated, fungal contamination is frequent unless containers are thoroughly cleaned with ethanol before each use. Addition of fungicides such as cycloheximide or nystatin to the medium is usually effective in reducing this problem.

A straightforward method for the isolation of *Methylocystis* and other methanotrophs uses NMS agar media containing a small amount of yeast extract (0.05% w/v) and methanol (0.05% v/v) (Malashenko et al., 1975), with incubation still occurring under a methane/air atmosphere. The enrichment culture is then serially diluted onto the media to the point of extinction. Single colonies on spread plates are then transferred to liquid media. A number of passages from liquid media to spread plates and back to liquid media are often required. The purity of the culture can be assessed by assuring that strains show no growth on nutrient media in the presence or absence of methane and that cells possess a reasonably consistent morphology. More details on enrichment, isolation, and the potential problems have been detailed by Whittenbury et al. (1970b) and Hanson et al. (1992).

Some *Methylocystis* strains, especially those of *M. echinoides*, grow poorly on agar-solidified media. Highly purified agars at lower concentrations may lead to improved growth. Alternatively, silica gel can be employed (Gal'chenko et al., 1975, 1977); however, silica gel is often difficult and time-consuming to prepare. For direct purification of these strains, an alternative approach involves serially diluting enrichments in NMS liquid media to

extinction in 96-well plastic titer trays (Bowman et al., 1997b). Several strains can be purified simultaneously in the same tray. After sufficient incubation, the wells of the highest dilutions showing growth are examined by microscopy. A number of separate transfers may be required to obtain morphologically homogeneous cultures.

MAINTENANCE PROCEDURES

Plate or slant cultures can be stored for several months at 4°C when stored under an atmosphere of 1:1 methane/air. Takeda (1988) found that the shelf life could be increased to over 12 months if cultures were kept under a 100% nitrogen atmosphere. *Methylocystis* strains are also amenable to cryopreservation (with 20% dimethylsulfoxide as a cryoprotectant) and freeze drying (with 20% skim milk or 10% horse serum as the cryoprotectant).

PROCEDURES FOR TESTING SPECIAL CHARACTERS

The acetylene reduction assay traditionally used for detecting nitrogenase activity cannot be used for methanotrophs growing on methane because of co-oxidation of ethylene by MMO and because of the toxicity of the acetylene. Nitrogenase can be detected in methanotrophs grown at low oxygen partial pressures (in semi-solid agar) on methanol or in pre-grown cells supplied with a suitable source of reducing power such as formate, hydrogen, or ethanol (Murrell and Dalton, 1983b; Toukdarian and Lidstrom, 1984a, b; Takeda, 1988).

Putative soluble methane monooxygenase activity in methanotrophs can be rapidly assessed using the naphthalene oxidation technique (Brusseau et al., 1990). The methanotroph to be tested is cultured in copper-free NMS liquid media. Glassware used for cultivation should be washed with 1% (v/v) nitric acid to remove contaminating copper ions. An equal volume of a saturated naphthalene solution (approximately 30 mg/l at 25°C) is added to a suspension of methanotrophic cells that have been degassed briefly with nitrogen to remove residual methane. The mixture is then incubated at room temperature for approximately 1 h. 20 μ l of a freshly prepared solution of 1% (w/v) tetraoized α -dianisidine (Fast Blue salt B) is then added and mixed. The presumptive presence of soluble methane monooxygenase is indicated by the immediate formation of a reddish semi-soluble diazo compound formed from α -naphthol, which can be measured by spectrophotometry at 540 nm. An adaptation of this procedure to test colonies on agar plates is also available (Graham et al., 1992).

DIFFERENTIATION OF THE GENUS *METHYLOCYSTIS* FROM OTHER GENERA

Characteristics differentiating *Methylosinus* from *Methylocystis* are given in Table BXII. α .136.

TAXONOMIC COMMENTS

Phylogenetic analysis based on 16S rRNA sequencing indicates that *Methylocystis* strains should be classified in the *Alphaproteobacteria* (Bratina et al., 1992; Brusseau et al., 1994) and that they are closely related to strains of the genus *Methylosinus*. Due to the poor quality of some sequences, it is difficult to make a clear delineation of *Methylocystis* species from *Methylosinus* species. The close phylogenetic relationship of the genera is supported by the similarity of nucleotide distribution width and asymmetry of DNA melting curves across the genera (De Ley, 1969; Gebers et al., 1985; Bowman et al., 1991b). However, *Methylocystis* strains have

TABLE BXII.α.136. Differential characteristics of the genera *Methylocystis* and *Methylosinus*^a

Characteristics	<i>Methylocystis</i>	<i>Methylosinus</i>
<i>Morphology:</i>		
Reniform to rod-like	+	—
Pyriform or vibrioid	—	+
Motility	—	+
Exospores	—	+
Lipid cysts, spinae	D	—

^aFor symbols see standard definitions.

genomes 30–50% larger than those of *Methylosinus* strains; *Methylocystis* genomes range from 4.4–5.6 Mb in size (Bowman et al., 1991b). The DNA base composition of *Methylocystis parvus* is 63–67 mol% G + C (T_m), while the values for *Methylocystis echinoides* are slightly lower (61–62 mol% G + C [T_m]) (Meyer et al., 1986; Lysenko et al., 1988; Bowman et al., 1991a, b). DNA–DNA hybridization studies indicate *M. parvus* and *M. echinoides* are genetically distinct, sharing only low levels of DNA hybridization, and that no significant levels of hybridization occur between *Methylocystis* and *Methylosinus* strains (Lysenko et al., 1988; Bowman et al., 1991a, b).

Although *Methylocystis* was originally described by Romanovskaya et al. (1978) based on the phenotypic groupings created

by Whittenbury et al. (1970b), the genus was not included in the Approved List of Bacterial Names (Skerman et al., 1980). Only *M. parvus* was included in the genus at the time, and several other species including “*Methylocystis methanolicus*” (Gal’chenko et al., 1977), “*Methylocystis pyreiformis*”, “*Methylocystis fuscus*” (Gal’chenko, 1977), and “*Methylocystis fistulosa*” (Meyer, 1977) were ignored owing to lack of data, lack of a type strain, and/or the possibility that some were still mixed cultures (D. Prauser, personal communication). The genus was subsequently revived and described in 1993 (Bowman et al., 1993c). Studies using protein electrophoresis (Gal’chenko and Nesterov, 1981) and serotyping (Bezrukova et al., 1983) still suggest that the diversity in *Methylocystis* is greater than what is currently recognized.

Strains designated *M. echinoides* by Gal’chenko et al. (1977) were very similar to the strain IC 493S/5 (IMET 10491) of Haubold (1978) according to protein electrophoretic patterns (Gal’chenko and Nesterov, 1981), and strain IMET 10491 subsequently became the type strain of *M. echinoides*. “*Methylocystis minimus*” IMET 10519 (Whittenbury et al., 1970b; Romanovskaya et al., 1978) is a subjective synonym of *M. parvus* (Bowman et al., 1993c), as it shares about 68% similarity by DNA hybridization with *M. parvus* strain OBBP. Strain A of “*Methylovibrio soehngenii*” (Hazeu and Steenis, 1970) probably should be described as a strain of *M. parvus* (Anthony, 1982).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *METHYLOCYSTIS*

Phenotypic characteristics differentiating the species *M. parvus* and *M. echinoides* are presented in Table BXII.α.137. Additional phenotypic characteristics for these species are presented in Table BXII.α.138.

TABLE BXII.α.137. Differential characteristics of the species of the genus *Methylocystis*^a

Characteristic	<i>M. parvus</i>	<i>M. echinoides</i>
<i>Morphology:</i>		
Reniform	+	—
Short rods	—	+
Spinae	—	+
Lipid cysts	+	—
Growth on agar	good	poor
Desiccation resistant	+	—
Growth at 37°C	+	—
Mol% G + C (T_m)	63–67	61–62

^aFor symbols see standard definitions.**TABLE BXII.α.138.** Other characteristics of the species of the genus *Methylocystis*^a

Characteristics	<i>M. parvus</i>	<i>M. echinoides</i>
Polyphosphate	d	—
Poly-β-hydroxybutyrate inclusions	+	+
Melanin-like pigments	d	+
pH range for growth	5.5–8.5	5.5–8.5
<i>Growth in the presence of:</i>		
0.02% NaN ₃	d	+
0.0075% KCN	+	+
0.001% crystal violet	—	—
0.001% malachite green	d	—
0.01% SDS	+	+
Lysed by 2% SDS	—	—
Oxidase/catalase activity	+	+
Phosphatase, urease	d	—
Triphenyltetrazolium chloride (TTC) reduction	—	—
Nitrogen fixation	+	+

^aFor symbols see standard definitions.List of species of the genus *Methylocystis*

- Methylocystis parvus*** (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}
par'vus. L. adj. *parvus* small.

Phenotypic characteristics are presented in the genus description, in the text, and in Tables BXII.α.137 and BXII.α.138.

Isolated from soil and fresh water sediments.

The mol% G + C of the DNA is: 63–67 (T_m).

Type strain: OBBP, ACM 3309, ATCC 35066, IMET 10483, NCIMB 11129.

GenBank accession number (16S rRNA): M29026, Y18945.

- Methylocystis echinoides*** (ex Gal’chenko, Shishkina, Suzina

and Trotsenko 1977) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}

ech.i.noi'des. Gr. adj. *echino* spiny, hedgehog-like; Gr. suff. *ides* similar to; M.L. neut. adj. *echinoides* spiny like a hedgehog.

Phenotypic characteristics are presented in the genus description, in the text, and in Tables BXII.α.137 and BXII.α.138.

Isolated from sewage and fresh water sediments.

The mol% G + C of the DNA is: 61–62 (T_m).

Type strain: IMET 10491, NCIMB 13100.

GenBank accession number (16S rRNA): L20848.

Additional Remarks: This sequence is not from the type strain.

Species Incertae Sedis

1. "**Methylocystis fistulosa**" Meyer 1977, 19.
2. "**Methylocystis fuscus**" Gal'chenko 1977, 15.
3. "**Methylocystis methanolicus**" Gal'chenko, Shishkina, Suzina and Trotsenko 1977, 726.

Deposited strain: 36, NCIMB 13101, IMET 10597.

4. "**Methylocystis pyreiformis**" Gal'chenko 1977, 15.

Deposited strain: NCIMB 13102.

GenBank accession number (16S rRNA): L20803.

Genus II. Albibacter Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2001, 1056^{VP}

NINA V. DORONINA AND YURI A. TROTSENKO

Al.bi.bac'ter. L. adj. *albus* white; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *baktron* a small rod; M.L. masc. n. *Albibacter* small, white rod.

Rods 0.9–1.0 × 1.2–1.8 μm, singly, in pairs, or in clusters. **Non-motile**. Gram negative. Multiplication is by binary fission. Endospores and prosthecae are not formed. Colonies are white. Obligately **aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Nitrate is reduced to nitrite. Growth occurs on nutrient agar and peptone–yeast extract–glucose (PYG) agar. **Facultatively methylo-trophic and chemolithotrophic. Assimilate C₁ compounds via the ribulose biphosphate pathway.** Able to grow on a wide spectrum of polycarbon substrates. Sugars are utilized aerobically but not fermented. Ammonium salts, nitrate, urea, peptone, some amino acids, and methylated amines are utilized as nitrogen sources. Growth factors are not required. Methyl red and Voges–Proskauer negative. Catalase and urease positive. **Oxidase activity is very low.** Indole is formed from L-tryptophan in the mineral medium with methanol as a sole carbon and energy source and with KNO₃ as the nitrogen source. The major ubiquinone is Q-10. The predominant cellular fatty acids are *cis*-vaccenic (C_{18:1 ω7}) and palmitic (C_{16:0}). The dominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, and phosphoserine. Belongs to the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 66.7.

Type species: **Albibacter methylovorans** Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2001, 1056.

FURTHER DESCRIPTIVE INFORMATION

Albibacter species are facultatively autotrophic and methylo-trophic. Good growth occurs on mineral salt medium with gas mixture of CO₂/H₂/O₂, dichloromethane (0.05–0.1%, v/v), methanol (0.5%, v/v), methylamine (0.3%, w/v), or formate (0.05%, w/v) as the carbon and energy sources. Dichloromethane-grown cells contain an inducible, reduced glutathione (GSH)-dependent dichloromethane dehalogenase, whereas methylamine- or methanol-grown cells possess the appropriate pyrroloquinoline quinone-linked dehydrogenases. Formaldehyde is further oxidized by a GSH-dependent formaldehyde dehydrogenase to formate. The latter is finally oxidized to CO₂ by formate dehydrogenase. The cells assimilate CO₂ via the ribulose biphosphate (RuBP) pathway and show phosphoribulokinase and ribulosebiphosphate carboxylase activity. Neither the serine nor the ribulose monophosphate pathway of C₁ assimilation is operative, due to the absence of the appropriate specific enzymes, namely hydroxypyruvate reductase (NADH), serine-glyoxylate aminotransferase, ATP malate lyase, and hexulosephosphate synthase, respectively.

The cells have no 2-keto-3-deoxy-6-phosphogluconate aldolase; therefore, the Entner–Doudoroff pathway is not functioning. In addition, the enzymes of the pentose phosphate pathway (glu-

cose-6-phosphate and 6-phosphogluconate dehydrogenases) possess low activities. Hence, fructose-1,6-bisphosphate aldolase plays an important role in metabolic conversions of phosphotrioses. The cells contain pyruvate dehydrogenase as well as a complete set of the citric acid cycle and glyoxylate shunt enzymes.

The primary assimilation of ammonia occurs by both reductive amination of α-ketoglutarate and by the glutamate cycle.

ENRICHMENT AND ISOLATION PROCEDURES

Albibacter methylovorans was isolated on dichloromethane agar from enrichment culture that had been inoculated with a groundwater sample (Doronina et al., 2001).

MAINTENANCE PROCEDURES

The bacteria can be stored in liquid mineral medium K, which has the following composition (g/l): KH₂PO₄, 2.0; (NH₄)₂SO₄, 2.0; NaCl, 0.5; MgSO₄·7H₂O, 0.125; FeSO₄·7H₂O, 0.002; pH 7.2. The cultures can be stored in the liquid medium at 4°C for at least one month and on agar slants at 4°C for 2–3 months. Cells can also be frozen in medium K containing 40% glycerol and stored at –70°C or lyophilized with a cryoprotectant (skim milk).

DIFFERENTIATION OF THE GENUS *ALBIBACTER* FROM OTHER GENERA

Albibacter methylovorans differs from the facultatively chemolithotrophic and methylo-trophic members of the genera *Xanthobacter*, *Blastobacter*, *Angulomicrobium*, *Ancylobacter*, and *Ralstonia* by morphological features. The representatives of *Xanthobacter* form pleomorphic cells, and their colonies are slimy and are yellow due to the water-insoluble carotenoid pigment, zeaxanthin dirhamno-zide. Species of the genera *Blastobacter* (irregular, ovoid, pleomorphic rods) and *Angulomicrobium* (tetrahedron-shaped cells) multiply by budding. The vibrioid cells of the genus *Ancylobacter* have a characteristic morphology and rings are formed occasionally prior to cell division. *Ralstonia* (formerly *Alcaligenes*) species are motile by one to eight peritrichous flagella.

TAXONOMIC COMMENTS

The levels of DNA similarity with representatives of *Xanthobacter*, *Blastobacter*, *Angulomicrobium*, *Ancylobacter*, and *Ralstonia* are less than 7%. Phylogenetic analysis by 16S rDNA sequencing (Doronina et al., 2001) confirmed the absence of close relatedness between *A. methylovorans* and the species of the genera *Xanthobacter* and *Blastobacter*. Although *A. methylovorans* resembles *Paracoccus* species in morphology, ubiquinone system, C₁-assimilation pathway, and range of mol% G + C content, it has very low DNA–DNA similarities (5–7%) with the type species of this genus

and thus belongs to another subgroup of the *Alphaproteobacteria*. Despite rather high 16S rDNA sequence similarity to *Methylophila capsulata*, *A. methylovorans* is clearly distinct from this serine pathway methylotroph by morphology, cellular phospholipids and fatty acids, autotrophic growth, and RuBP pathway operation, as

well as by a very low DNA–DNA similarity. In the phylogenetic tree derived from 16S rDNA sequences, the genus *Albibacter* forms a distinct branch within the *Alphaproteobacteria* (Doronina et al., 2001).

List of species of the genus *Albibacter*

1. ***Albibacter methylovorans*** Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2001, 1056^{VP}
me.thy.lo.vor'ans. Fr. *méthyle* the methyl group; L. part. adj. *vorans* devouring, digesting; *methylovorans* digesting methyl groups.

The characteristics are as described for the genus, with the following additional features.

Fig. BXII.α.154 illustrates the morphological features.

Colonies on methanol or peptone agar are circular and 1–2 mm in diameter, white, convex, translucent to opaque, and mucoid. Growth occurs at 10–35°C and pH 6.0–9.0. Optimal conditions for growth are 28–30°C, pH 7.5–8.0. No growth occurs in the presence of 3% NaCl. Starch is hydrolyzed but not gelatin or cellulose.

Utilizable carbon sources are dichloromethane, methanol, methylamine, formate, CO₂/H₂, D-glucose, D-fructose, D-mannose, L-arabinose, D-xylose, sorbose, ribose, maltose,

sucrose, D-sorbitol, D-mannitol, inositol, glycerol, dulcitol, adonitol, ethanol, acetate, α-ketoglutarate, fumarate, pyruvate, succinate, malate, citrate, oxaloacetate, propionate, *cis*-aconitate, L-glutamate, L-alanine, and acetamide. Not utilizable are methane, chloromethane, formaldehyde, di- and trimethylamines, thiocyanate, thiosulfate, dimethylsulfoxide, and dimethylacetamide. Yeast extract (0.01%), or a mixture of biotin and thiamin (both at 20 µg/l), stimulates growth.

Resistant to penicillin and rifampicin, but sensitive to gentamicin, kanamycin, ampicillin, neomycin, novobiocin, nalidixic acid, tetracycline, and lincomycin.

The type (and only) strain was isolated from groundwater in Switzerland.

The mol% G + C of the DNA is: 66.7 (*T_m*).

Type strain: DM10, DSM 13819, VKM B-2236.

GenBank accession number (16S rRNA): AF273213.

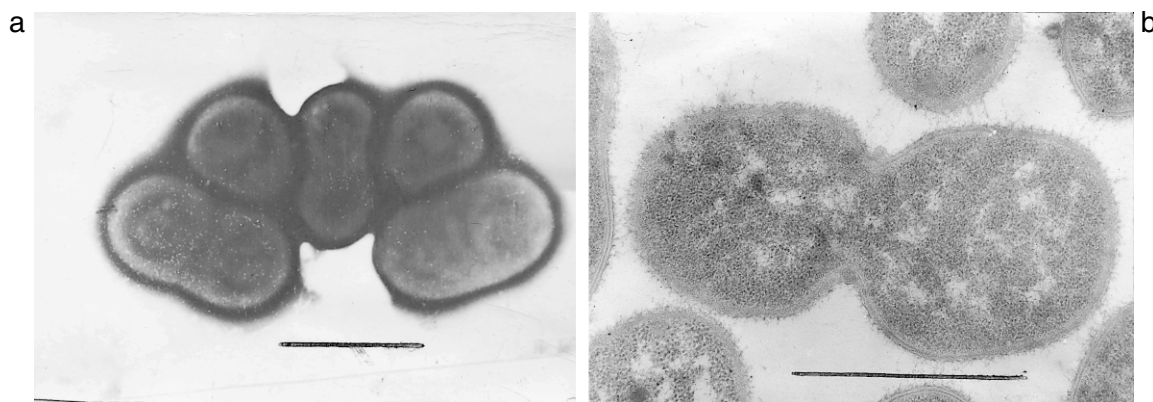


FIGURE BXII.α.154. *Albibacter methylovorans* (a) Negatively stained cells. (b) Ultrathin sections showing cell wall structure. Bars = 1 µm.

Genus III. *Methylosinus* (ex *Romanovskaya*, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}

JOHN P. BOWMAN

Me.thyl.o.si' nus. Fr. *méthyle* the methyl radical; *sinus* bend; M.L. n. *Methylosinus* methyl bender.

Cells are **pyriform or vibrioid** in shape, 0.5–1.5 × 1.5–3.0 µm, usually arranged in rosettes of 4–6 cells. Reproduce by **binary and budding division**. In budding division, the bud contains a **heat and desiccation-resistant exospore**, which germinates into a vegetative daughter cell; this **daughter cell is motile**, propelled by a tuft of 3–10 polar and/or subpolar flagella. Cells contain **type II intracytoplasmic membranes** that are arranged as multiple layers along the periphery of the cell wall. Strongly accumulate inclusions of poly-β-hydroxybutyrate. Aerobic, possessing a **strictly respiratory type of metabolism with oxygen as the terminal electron acceptor**. **Obligately methanotrophic**, utilizing

only methane and methanol as sole carbon and energy sources. C₂₊ and other C₁ compounds are not utilized. Fix formaldehyde for cell carbon via the serine pathway. Enzymes for the Benson–Calvin cycle pathway are absent. The tricarboxylic acid pathway is complete. **Fix atmospheric nitrogen by means of an oxygen-sensitive nitrogenase**. Mesophilic. Nonhalophilic. Optimal temperature, 25–30°C; optimal pH, 6.5–7.0. Major habitats include soil, freshwater sediments and groundwater. **Primary fatty acids are C_{18:1} ω_{8c} and C_{18:1} ω_{7c}**. **Primary quinone is ubiquinone-8 (Q-8)**. Member of the Family *Methylocystaceae* found in the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 62–67 (T_m).

Type species: *Methylosinus trichosporium* (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751.

FURTHER DESCRIPTIVE INFORMATION

Morphology and exospore formation Cells of *Methylosinus trichosporium* have a pear-shaped (pyriform) morphology with a width of 0.5–1.5 μm and a length of 1.5–3 μm . *Methylosinus sporium* cells have a similar size but are vibrioid. When grown in liquid media, both species form rosettes consisting of 4–6 cells. Photomicrographs of the morphology of *Methylosinus* species have been published by Whittenbury et al. (1970a, b). In logarithmic phase, the cells divide by standard binary division. As cultures enter the stationary growth phase, an increasing proportion of cells begin to sporulate and reproduce by budding, either when the cells are arranged in rosettes or singly. The bud that is formed consists of an exospore surrounded by a part of the cell wall. A sporulating cell ceases to divide or bud, becomes granulated, and eventually lyses. It will often produce an extensive fibrillar capsule, which is not formed by vegetative cells (Whittenbury et al., 1970a). Exospore formation by *M. trichosporium* is stimulated by oxygen limitation (Reed and Dugan, 1978). When spores appear, they are initially non-refractile, Gram-negative, coccoid bodies at the end of sporulating cells. As the spores mature, they become increasing refractile and acid-fast. The malachite green spore stain (Doetsch, 1981) can be used to visualize mature spores. Thin sections show that the exospore consists of an electron-dense outer coat (exosporium) surrounded by a cell wall derived from the parent cell. The *M. trichosporium* exospores possess a capsular coat that is attached to the exosporium but is distinct from the parent cell's capsular layer (Reed et al., 1980). Within the exospore, there is a laminated inner coat and a poorly defined cortex (Reed et al., 1980; Titus et al., 1982). Dipicolinic acid and respiratory activity are not detectable in the mature exospores. The exospores are resistant to desiccation and can survive 18 months of drying. Exospores can also withstand heating at 85°C for 10 min and 10 min of ultrasonication. The exospores are structurally similar to those formed by *Rhodomicrobium vannielii* (Titus et al., 1982). When freshly formed, exospores take ~2–3 d to germinate, whereas heat- or desiccation-treated exospores may take weeks to recover. During the germination process, which lasts 5–7 h, the exospores lose their refractility and shed the capsular coats (if any). The exosporium then opens to release a short rod-like daughter cell, which rapidly becomes motile by forming a tuft of 3–10 unsheathed flagella. This cell usually undergoes a few generations of binary division before settling into a rosette to commence exospore production.

Cell walls *Methylosinus* strains produce poly- β -hydroxybutyrate as an internal carbon reserve (Weaver et al., 1975b; Best and Higgins, 1981). The cell walls are of the usual Gram-negative type; however, unlike most other Gram-negative bacteria, *Methylosinus* strains are very resistant to lysis by detergents (such as sodium dodecyl sulfate) and to lytic bacteria (Bowman et al., 1993c; Starostina and Pashkova, 1993). Cell lysis can be caused by a lysozyme pretreatment (1 mg/ml lysozyme at 37°C, for 30–60 min), followed by addition of 2% sodium dodecyl sulfate.

Cultural characteristics *Methylosinus* strains grow abundantly in nitrate mineral salts broth and on media solidified by all brands of agar (see *Methylocystis* for medium formula). The colonies of *M. trichosporium* and *M. sporium* are similar: circular,

convex, with an entire edge and creamy consistency. Colonies typically reach 1–2 mm in diameter on NMS agar after 7 d incubation. One strain (IMV B-3037), identified as a strain of *M. sporium*, forms brilliant red nondiffusible pigments that have a slight green metallic sheen. Spectral analysis suggests they are similar to the prodigiosin pigments of *Serratia marcescens*. Two different pigment types were detected and are referred to as methylosin A and B (Strauss and Berger, 1983).

Methylosinus strains are mesophilic and neutrophilic with growth occurring from 10 to 40°C and pH 5.5–9.0. Optimal growth occurs at approximately 30°C and at pH 6.5–7.0. Most strains will either not grow or grow only poorly in the presence of NaCl concentrations greater than 0.3 M. No strains have been found to possess growth factor requirements.

Nutrition and metabolism *Methylosinus* strains are strictly aerobic and obligately methanotrophic. The only carbon and energy sources supporting growth are methane and methanol (Meyers, 1982; Bowman et al., 1993c). Formaldehyde is assimilated by cells for cell carbon via the serine pathway (Wolfe and Higgins, 1979). All strains possess a complete tricarboxylic acid cycle containing a NADP-dependent isocitrate dehydrogenase (Colby et al., 1979). Strains can grow over a wide range of oxygen concentrations (<0.5–60%, v/v) and are not microaerophilic as has been suggested in some previous studies. Only when oxygen levels drop below 0.5% does growth become limiting (Ren et al., 1997). Interestingly, *M. trichosporium* OB3b can survive anoxia for several months (presumably due to exospore formation) and can rapidly respond when methane and oxygen once again become available (Roslev and King, 1994, 1995). Oxygen limitation results in a reduction in the amount of intracytoplasmic membrane (ICM) formation (Scott et al., 1981). When grown under either methane or nitrogen limitation, cells also appear to lack the typical paired type II ICMs, instead having membrane vesicles (Best and Higgins, 1981; Scott et al., 1981). All strains produce catalase and cytochrome *c* oxidase. Nitrate and ammonia salts, amino acids, yeast extract and Casamino acids (Difco) can be used as sources of nitrogen (Warner et al., 1983; Toukdarian and Lidstrom, 1984b; Bowman et al., 1993c). Ammonia and nitrate are metabolized by the glutamine synthetase-glutamine 2-oxoglutarate aminotransferase system (Shishkina and Trotsenko, 1979; Murrell and Dalton, 1983a; Toukdarian and Lidstrom, 1984b). In addition, *Methylosinus* species are capable of fixing atmospheric nitrogen by means of an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b), which in some *Methylosinus* strains shows homology with the *nifH* genes of *Klebsiella pneumoniae* (Toukdarian and Lidstrom, 1984a; Oakley and Murrell, 1993).

To date, all strains tested form soluble methane monooxygenase (sMMO), which can be detected using the naphthalene oxidation assay (Brusseau et al., 1990), by a gene probe (Staintorpe et al., 1991), or by PCR detection of sMMO genes by means of specific primers (Holmes et al., 1995b; McDonald et al., 1995). Many studies have focused on the capability of *M. trichosporium* OB3b to co-oxidize a wide range of carbon substrates by its production of sMMO. The compounds oxidized are too many to list but include a wide range of aliphatic, heterocyclic, and aromatic compounds (Burrows et al., 1984). Several studies have focused on the industrial applications of this biocatalytic ability, particularly in the production of epoxides for plastics manufacture (Hou, 1984). Another focus of research has been in the bioremediation field. *M. trichosporium* OB3b and other similar strains can co-metabolize a wide range of chlorinated

aliphatic compounds, including major groundwater pollutants such as trichloroethylene, chloroform, and tetrachloroethylene (Oldenhuis et al., 1989; Tsien et al., 1989; Castro et al., 1996; Hamamura et al., 1997; Moran and Hickey, 1997; Tartakovsky et al., 1998). However, the industrial application of methanotrophs has been hampered by their relatively slow growth and their requirement for methane, which is a potentially explosive substrate that also competitively inhibits cometabolic reactions. Another problem is that trace copper levels can suppress sMMO activity, thereby eliminating or reducing transformation rates (Oldenhuis et al., 1989; Lontoh and Semrau, 1998). To overcome this, constitutive mutants lacking sMMO activity have been created by chemical mutagenesis and by marker exchange (Phelps et al., 1992; Fitch et al., 1993; Martin and Murrell, 1995; Tellez et al., 1998). These mutants can cometabolize trichloroethylene in the presence of high levels of copper. Some of these copper-tolerant mutants have been used for the development of treatment strategies for chlorinated aliphatic pollutants (Tschantz et al., 1995). In addition the sMMO gene cluster has been successfully cloned into *Pseudomonas putida* strain F1, which is not only able to degrade trichloroethylene but also possesses the ability to grow rapidly; moreover, it lacks the problem associated with methane competitive inhibition (Jahng and Wood, 1994; Jahng et al., 1996).

Plasmids Strains of *M. trichosporium* possess three large plasmids of cryptic function, which are 147 kb, 150 kb, and 160 kb in size, respectively. *M. sporium* ATCC 35069 has two cryptic plasmids, each around 100 kb in size. Restriction endonuclease analysis has indicated that different *M. trichosporium* strains possess identical plasmids; however, Southern blot hybridization analysis indicated that these plasmids share negligible homology with the plasmids of *M. sporium* (Lidstrom and Stirling, 1990).

DNA composition *Methylosinus* strains possess DNA base composition values of 62–67 mol% G + C (T_m). *M. trichosporium* strains possess values of 62–63 (T_m), while the values for *M. sporium* are slightly higher, at 65–67 (T_m) (Table BXII.α.139). The characteristics of the DNA melting curves support a close relationship between *Methylosinus* and *Methylocystis*, as they possess similar nucleotide distribution widths and curve asymmetries (De Ley, 1969; Gebers et al., 1985; Bowman et al., 1991b). The genome size of various *Methylosinus* strains, as calculated by renaturation kinetics, is in the range of 3.0–3.5 Mb, somewhat smaller than that of *Methylocystis* species (Bowman et al., 1991b).

Protein analysis Analysis of protein banding patterns has shown *M. sporium* and *M. trichosporium* to be similar, sharing about 60% of the bands (Gal'chenko and Nesterov, 1981). Only low levels of serological cross reactivity have been detected between antisera raised from *M. trichosporium* and *M. sporium* (Bezrukova et al., 1983).

TABLE BXII.α.139. Differentiation of the species of the genus *Methylosinus*^a

Characteristics	<i>M. trichosporium</i>	<i>M. sporium</i>
<i>Morphology:</i>		
Pyriform	+	–
Vibrioid	–	+
Melanin-like pigments	–	d
Phosphatase, urease	–	+
Mol% G + C of the DNA	62–63	65–67

^aFor symbols see standard definitions.

Lipids *Methylosinus* species possess phospholipid fatty acids that consist mainly of C_{18:1 ω8c}, C_{18:1 ω7c}, and C_{16:1 ω7c}, with smaller amounts of C_{18:0} and cyclopropane fatty acids (Weaver et al., 1975b; Makula, 1978; Nichols et al., 1985; Bowman et al., 1991b; Guckert et al., 1991) (Table BXII.α.136). The profile is very similar to that of *Methylocystis* and differs only in possessing a greater abundance of C_{16:1 ω7c} and a lower level of C_{18:0}. The fatty acid C_{18:1 ω8c} is an unusual feature that is practically unique to the family *Methylocystaceae* (Nichols et al., 1985; Bowman et al., 1991b). In ecological studies, C_{18:1 ω8c} has become a very useful signature for the detection and quantification of type II methanotroph populations in environmental samples (Nichols et al., 1985, 1987; Bowman et al., 1991a, b; Guckert et al., 1991; Ross et al., 1997; Sundh et al., 1997). In addition, major lipopolysaccharide-derived hydroxy fatty acids include C_{14:0 3OH}, C_{16:0 3OH}, and C_{18:0 3OH} (Bowman et al., 1991a; Skerratt et al., 1992). In addition, *Methylosinus sporium* possesses unusual ω-1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992) that make up about 15% of the total hydroxy fatty acids. These hydroxy acids have been previously detected in freshwater sediments (Mendoza et al., 1987), and *Methylocystis* and *Methylosinus* represent the first recognized biological source of these particular lipids.

The neutral sugar components of the LPS oligosaccharide core in *M. trichosporium* OB3b include mostly rhamnose, glucose, and heptose (Sutherland and Kennedy, 1986). *Methylosinus* species contain a suite of phospholipids (Makula, 1978; Andreev and Galchenko, 1983), including (with relative % levels): phosphatidylidimethylethanolamine (50%), phosphatidylglycerol (13%), phosphatidylmethylethanolamine (20%), phosphatidylcholine (10%), and lysophosphatidylglycerol (5%). The major quinone in *Methylosinus* is Q-8 (Collins and Green, 1985). Neunlist and Rohmer (1985) found that *M. trichosporium* OB3b contained two free triterpenoids of the hopane family, including an aminotriol (35-aminobacteriohopane-32,33,34-triol) and a novel tetrol (35-aminobacteriohopane-31,32,33,34-tetrol). The aminotriol has been observed previously in *Rhodomicoccus vannielii* (Neunlist and Rohmer, 1985).

Ecology *Methylosinus* strains have been isolated from a variety of soil and freshwater sediment habitats with *M. sporium* being more frequently isolated than is *M. trichosporium* (Bowman, 1992). *Methylosinus* dominates the culturable methanotroph population from groundwater (Bowman et al., 1993a) and has been isolated from the root systems of various aquatic macrophytes (Calhoun and King, 1998). Indirect immunofluorescence studies give a rough indication of the presence of *Methylosinus* species and indicate that *Methylosinus* species congregate at high populations in surface sediments of various freshwater and brackish water bodies (Reed and Dugan, 1978; Abramochkina et al., 1987; Malashenko et al., 1987; Gal'chenko et al., 1988; Gal'chenko, 1994). *Methylosinus* also occurs in rice paddy soils (Saralov and Babnazarov, 1982) and has been detected by gene probe in blanket peat bogs (McDonald et al., 1996).

ENRICHMENT AND ISOLATION PROCEDURES

Procedures for enrichment and isolation are the same as described for the genus *Methylocystis*.

MAINTENANCE PROCEDURES

Methods for preservation and storage of strains are the same as described for the genus *Methylocystis*.

DIFFERENTIATION OF THE GENUS *METHYLOSINUS* FROM OTHER GENERA

Characteristics differentiating *Methylosinus* from *Methylocystis* are given in Table BXII.α.136.

TAXONOMIC COMMENTS

Phylogenetic studies place *Methylosinus* within the *Alphaproteobacteria*. The genus is very closely related to *Methylocystis* and is more distantly related to an assemblage of facultatively methylotrophic, nitrogen-fixing, and phototrophic bacteria, including *Azorhizo-*

bium, *Xanthobacter*, *Ancylobacter*, *Blastochloris*, *Rhodoplanes*, *Rhodopseudomonas viridis*, *Rhodopseudomonas rosea*, *Rhodopseudomonas acidophila*, and *Beijerinckia*. Owing to the relatively poor quality of some of the 16S rRNA sequences, the distinction between *Methylosinus* and *Methylocystis* based on phylogeny alone is ambiguous.

No significant DNA–DNA hybridization (<25%) exists between *Methylosinus trichosporium* and *Methylosinus sporium* strains and with various strains of *Methylocystis* (Lysenko et al., 1988; Bowman et al., 1993c).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *METHYLOSINUS*

Characteristics differentiating *M. trichosporium* and *M. sporium* are shown in Table BXII.α.139. Additional phenotypic characteristics for these species are presented in Table BXII.α.140.

List of species of the genus *Methylosinus*

- Methylosinus trichosporium*** (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}
tri.cho.spo'ri.um. Gr. n. *trix* hair; L. n. *spora* spore; *trichosporium* hair spore-former.

Phenotypic characteristics are presented in Tables BXII.α.139 and BXII.α.140.

Isolated from soil, fresh water sediments, and groundwater.

The mol% G + C of the DNA is: 62–63 (*T_m*).

Type strain: OB3b, ACM 3311, ATCC 35070, IMET 10543, NCIMB 11131.

GenBank accession number (16S rRNA): M29024, Y18947.

- Methylosinus sporium*** (ex Romanovskaya, Malashenko and Bogachenko 1978) Bowman, Sly, Nichols and Hayward 1993c, 751^{VP}
spo'ri.um. M.L. n. *sporum* spore-former.

Phenotypic characteristics are presented in Tables BXII.α.139 and BXII.α.140.

Isolated from soil and fresh water sediments.

The mol% G + C of the DNA is: 65–67 (*T_m*).

Type strain: 5, ACM 3306, ATCC 35069, IMET 10545, NCIMB 11126.

GenBank accession number (16S rRNA): Y18946.

TABLE BXII.α.140. Other characteristics of the species of the genus *Methylosinus*^a

Characteristics	<i>M. trichosporium</i>	<i>M. sporium</i>
Poly-β-hydroxybutyrate inclusions	+	+
Heat and desiccation resistance	+	+
pH range for growth	5.5–9.0	5.5–9.0
Growth at 37°C	+	+
Growth in the presence of:		
0.02% NaN ₃	+	+
0.0075% KCN	+	+
0.001% crystal violet	–	d
0.001% malachite green	+	+
0.01% SDS	+	+
3% NaCl	–	–
Lysed by 2% SDS	–	–
Oxidase/catalase activity	+	+
Triphenyltetrazolium chloride (TTC) reduction	–	–
Nitrogen fixation	+	+

^aFor symbols see standard definitions.

Genus *Incertae Sedis* IV. ***Methylopila*** Doronina, Trotsenko, Krausova, Boulygina and Tourova 1998a, 1319^{VP}

NINA V. DORONINA AND YURI A. TROTSENKO

Me.thy.lo.pi'la. Fr. *méthyle* the methyl radical; Gr. adj. *pila* ball or sphere; M.L. *Methylopila* methyl-using sphere.

Rods, 0.5–0.7 × 1.0–1.3 μm, occurring singly or in pairs. **Motile or nonmotile**. If motile, the cells have a single lateral flagellum. Gram negative. **Poly-β-hydroxybutyrate granules are formed**. Prosthecae are not formed. Colonies are white. Do not produce pyocyanine and fluorescein. Methyl red and Voges–Proskauer negative. **Oxidase and urease positive**. Indole is formed from L-tryptophan on the mineral medium with methanol as the sole carbon and energy source and KNO₃ as the sole nitrogen source. Produce acid from sugars oxidatively. **Aerobic**, having a strictly

respiratory type of metabolism with oxygen as the terminal electron acceptor. Chemoorganotrophic. **Facultatively methylo-trophic. Assimilate C₁ compounds by the serine pathway (isocitrate lyase[–] variant)**. Growth factors are not required. Optimal pH 6.5–7.5; no growth above pH 9 or below pH 5. Optimal temperature, 28–35°C; no growth at 42°C. No growth with 7% NaCl. NaCl is not required for growth. Cellular fatty acid profile is characterized by the presence of 45–70% *cis*-vaccenic acid (C_{18:1 ω7}); main quinone is Q-10. Dominant cell phospholipids

are phosphatidylethanolamine and phosphatidylcholine. The genus belongs to the order *Rhizobiales* in the class *Alphaproteobacteria*.

The mol% G + C of the DNA is: 66–70.

Type species: *Methylopila capsulata* Doronina, Trotsenko, Krausova, Boulygina and Tourova 1998a, 1320.

FURTHER DESCRIPTIVE INFORMATION

After division, the cells remain connected by a constriction that appears to be formed by the outer membrane.

The serine-pathway-specific enzymes (hydroxypyruvate reductase, serine-glyoxylate aminotransferase, malate lyase) are present. Isocitrate lyase is absent.

Primary assimilation of ammonia occurs both by reductive amination of α -ketoglutarate and via the glutamate cycle.

ENRICHMENT AND ISOLATION PROCEDURES

Methylopila capsulata strains can be isolated from soil samples as colonies on the mineral base medium K¹ with 2% (w/v) of Difco agar and 1% methanol (Doronina et al., 1998a). *Methylopila helvetica* strains can be isolated from groundwater and active sludge as dichloromethane-utilizing bacteria on the medium DM². Wa-

ter and sludge samples (0.5 g) (Gälli and Leisinger, 1985) are used for direct inoculation of 10 ml DM medium in closed 50 ml flasks containing 100 μ mol CH₂Cl₂. Enrichment cultures are grown at 30°C for 5 d on a rotary shaker and those acidifying the medium are then transferred to fresh medium. After three transfers, the enrichment cultures are then plated into Petri dishes with solidified medium containing 2% (w/v) of Difco agar and 0.01% (w/v) of bromothymol blue, and incubated in a desiccator with 0.05% (v/v) CH₂Cl₂ in the gas phase; pure cultures can then be isolated from individual colonies that change the medium color.

MAINTENANCE PROCEDURES

Strains may be maintained on agar slants at 4°C for at least 2 months. Lyophilization may be used for long-term preservation, with skim milk as a cryoprotectant.

DIFFERENTIATION OF THE GENUS *METHYLOPILA* FROM OTHER GENERA

The major characteristics differentiating the genus *Methylopila* from other facultative serine pathway methylobacteria are summarized in Table BXII.α.63 in Genus *Methylarcula*.

TAXONOMIC COMMENTS

In the phylogenetic tree derived from 16S rRNA sequences, the genus *Methylopila* forms a distinct branch within the order *Rhizobiales* of the class *Alphaproteobacteria*.

Methylopila species share less than 10% DNA–DNA similarity with members of *Methylorhabdus*, *Methylobacterium*, *Aminobacter*, *Hyphomicrobium*, *Methylarcula*, and *Pseudomonas*.

1. K medium contains (g/l of distilled water): (NH₄)₂SO₄, 2.0; KH₂PO₄, 2.0; NaCl, 0.5; MgSO₄·7H₂O, 0.125; FeSO₄·7H₂O, 0.002. The pH of the medium is adjusted to 7.2 prior to autoclaving. Methanol is added to a final concentration of 1% (v/v).

2. DM medium contains (g/l of distilled water): KH₂PO₄, 1.36; Na₂HPO₄, 2.13; (NH₄)₂SO₄, 2.0; MgSO₄·7H₂O, 0.2. Prior to autoclaving, the pH of the medium is adjusted to 7.2. After sterilization, 1 liter of the medium is supplemented with 1 ml of a trace element solution containing (g/l): MnSO₄·5H₂O, 1.0; H₃BO₃, 1.0; CaCl₂, 0.25; ZnCl₂, 0.25; NH₄VO₃, 0.1; CoCl₂·6H₂O, 0.25 and Ca(NO₃)₂, 25.

List of species of the genus *Methylopila*

1. *Methylopila capsulata* Doronina, Trotsenko, Krausova, Boulygina and Tourova 1998a, 1320^{VP}
cap.su.la'ta. L. n. *capsule* a small chest, capsule; M.L. adj. *capsulata* having a capsule.

Pleomorphic rods or cocci, occurring singly or in pairs, having capsules covered in spikes. Motile by means of a single lateral flagellum. Fig. BXII.α.155a and b illustrate the morphological features. Colonies on nutrient agar or glucose–potato agar are round, viscous, semitransparent, convex, with even edges, 2 mm in diameter. Nitrates are reduced to nitrites slowly. Catalase activity is weak. H₂S is produced during growth in nutrient broth. The following carbon sources are used: methanol, mono-, di-, and trimethylamine, butanol, ethanol, glycerol, maltose, sucrose, L-arabinose, D-fructose, D-glucose, succinate, fumarate, and pyruvate. Weak hydrolysis of gelatin and starch occurs. The following nitrogen sources are used: ammonium salts, nitrate, urea, peptone, some amino acids, and methylated amines. Methylamine is oxidized to formaldehyde by amine dehydrogenase.

The type strain was isolated from soil of Tashkent City, Uzbekistan.

The mol% G + C of the DNA is: 67.2 (*T_m*).

Type strain: IM1, ATCC 700716, VKM B-1606.

GenBank accession number (16S rRNA): AF004844.

2. *Methylopila helvetica* Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2000c, 1953^{VP} (Effective publication: Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2000b, 216.)
hel.ve'ti.ca. M.L. neut. adj. *helvetica* from Helvetia, an old name of Switzerland.

Short rods or cocci, occurring singly or rarely in pairs. Motile by means of a single lateral flagellum. Fig. BXII.α.155c and d illustrate the morphological features. No growth occurs on nutrient agar or peptone–yeast extract–glucose agar. Colonies on methanol–salt agar are white, round, viscous, semitransparent, convex, 1–3 mm in diameter. Nitrate is reduced to nitrite. H₂S and NH₃ are not formed. Catalase positive. The following carbon sources are used: dichloromethane, methanol, formate, mono- and trimethylamine, ethanol, glycerol, pyruvate, fumarate, and succinate. Gelatin and starch are not hydrolyzed. Ammo-

nium salts, nitrate, methylated amines, and some amino acids are utilized as nitrogen sources. Methylamine is oxidized to formaldehyde by the *N*-methylglutamate pathway enzymes γ -glutamylmethylamide synthetase and *N*-methylglutamate synthase/lyase.

The type strain was isolated from groundwater in Switzerland.

The mol% G + C of the DNA is: 68.5 (T_m).

Type strain: DM9, CIP 106788, VKM B-2189.

GenBank accession number (16S rRNA): AF227126.

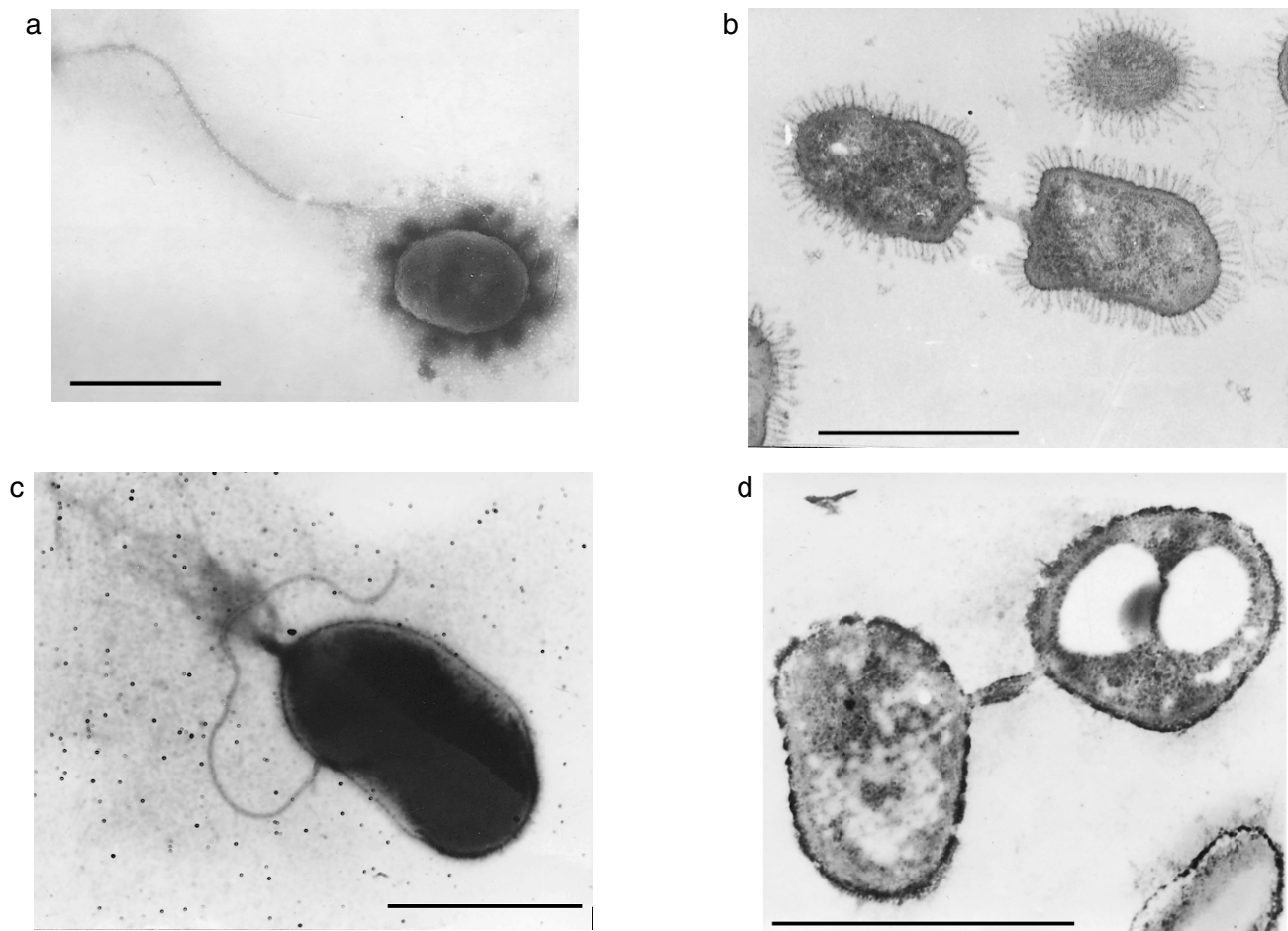


FIGURE BXII.α.155. *Methylopila capsulata* (a, b) and *Methylopila helvetica* (c, d). a and c, negatively stained cells, note the subpolar location of the single flagellum; a, the large amount of capsular material is evident. b and d, ultrathin sections; constriction in the final stage of cell division and granules of PHB. Bars = 1 μ m.

Family VI. *Beijerinckiaceae* fam. nov.

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Beij.e.rinck.i.a'ce.ae. M.L. fem. n. *Beijerinckia* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Beijerinckiaceae* the *Beijerinckia* family.

The family *Beijerinckiaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Beijerinckia* (type genus), *Chelatococcus*, *Methylocapsa*, and *Methylocella*. *Methylocapsa* was proposed after the

cut-off date for inclusion in this volume (June 30, 2001) and is not described here (see Dedysh et al. (2002)).

Aerobic. Form poly- β -hydroxybutyrate granules. Family is metabolically diverse. *Beijerinckia* and *Methylocella* fix nitrogen.

Type genus: ***Beijerinckia*** Derx 1950a, 145.

Genus I. Beijerinckia Derx 1950a, 145^{AL}

CHRISTINA KENNEDY

Beij.e.rinck' i.a. M.L. fem. n. *Beijerinckia* named after M.W. Beijerinck, the Dutch microbiologist (1851–1931).

Straight or slightly curved rods, $\sim 0.5\text{--}1.5 \times 7\text{--}4.5 \mu\text{m}$, with rounded ends. Cells occur singly or appear as dividing pairs. Sometimes large, misshapen cells $3.0 \times 5.0\text{--}6.0 \mu\text{m}$ occur; these are occasionally branched or forked. Intracellular granules of **poly- β -hydroxybutyrate (PHB)** are formed, generally one at each pole. Cysts (enclosing one cell) and capsules (enclosing several cells) may occur in some species. Gram negative. Motile by peritrichous flagella or nonmotile. **Aerobic**, having a strictly respiratory type of metabolism. **N₂ is fixed under aerobic or micro-aerobic conditions**. Optimal temperature for growth, 20–30°C; no growth occurs at 37°C. **Growth occurs between pH 3.0 and pH 9.5–10.0**. Liquid cultures can become a highly viscous, semitransparent mass; in some species the whole medium becomes opalescent and turbid, and adhering slime is not produced. **On agar media, especially under N₂-fixing conditions, copious slime is produced** and giant colonies with a smooth, folded, or plicate surface develop; some strains form slime having a more granular consistency. **Catalase positive**. **Glucose, fructose, and sucrose are utilized** by all strains and oxidized to CO₂. No growth occurs on peptone medium. Glutamate is utilized poorly or not at all. Species are found in soils, particularly **tropical regions**.

The mol% G + C of the DNA is: 54.7–60.7.

Type species: *Beijerinckia indica* (Starkey and De 1939) Derx 1950a, 146 (*Azotobacter indicum* (sic) Starkey and De 1939, 337.)

FURTHER DESCRIPTIVE INFORMATION

Cell morphology The typical microscopic appearance of *Beijerinckia* cells is shown in Figs. BXII.α.156 and BXII.α.157. Cells may be bicellular due to crosswall formation in the middle of the longitudinal direction of the cell (Figs. BXII.α.158 and



FIGURE BXII.α.157. *Beijerinckia indica* electron micrograph of a thin section showing the two polar lipid bodies, which are surrounded by a membrane ($\times 33,300$).

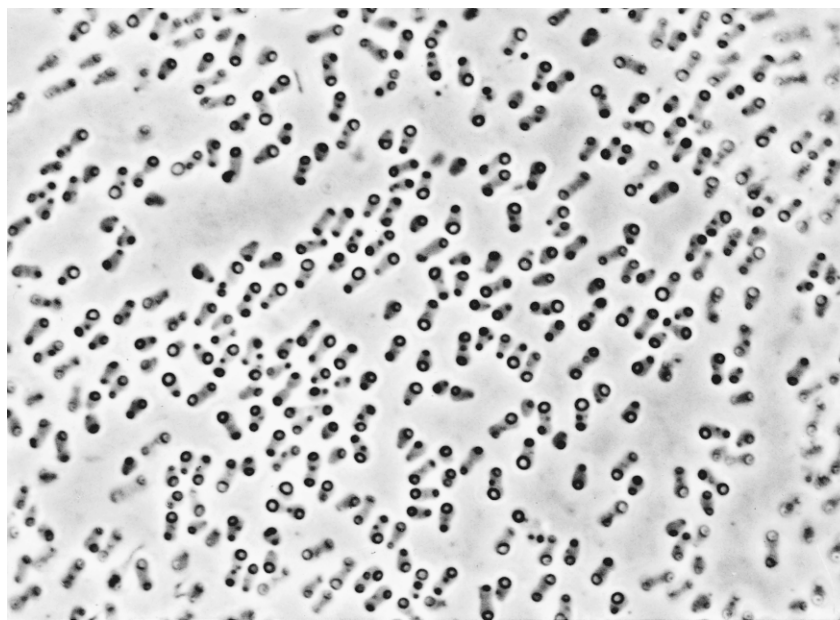


FIGURE BXII.α.156. *Beijerinckia indica* cells cultured on nitrogen-free glucose mineral agar (pH 5.0). The typical appearance of the cells and their intracellular polar lipid bodies is illustrated. Living preparation, phase-contrast microscopy ($\times 1500$).

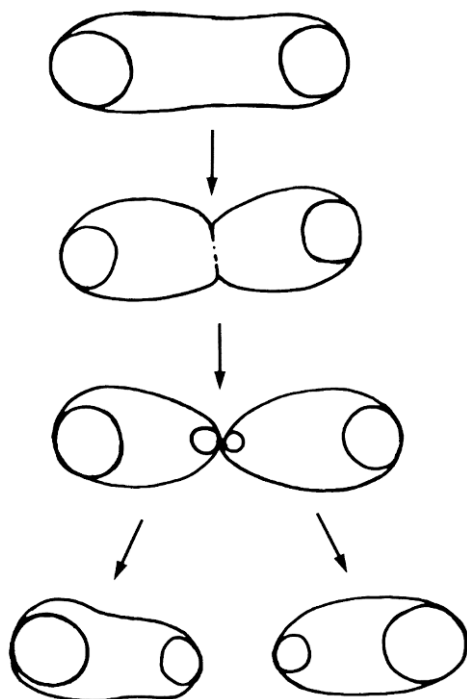


FIGURE BXII.α.158. Diagram of the life cycle of a *Beijerinckia* cell. A cell in division forms a crosswall in the middle of the longitudinal axis of the cell. In actively dividing cells, intermediate stages can often be seen.

BXII.α.159). Under certain cultural conditions, some *Beijerinckia* strains show coccoid cells without terminal lipid globules (Fig. BXII.α.160). Sometimes, especially in *B. mobilis* strains, more than two lipid globules per cell occur (Fig. BXII.α.162). The lipid material is poly-β-hydroxybutyrate (PHB) (Becking, 1974, 1984b). Cyst and capsule formation occur in some species (*B. fluminensis*, *B. mobilis*, and *B. indica*) (Figs. BXII.α.161, BXII.α.162, and BXII.α.163).

The flagella of motile cells are peritrichous in those strains studied (Thompson and Skerman, 1979). Flagella appear to originate from one-half of the often dumbbell-shaped cells. The wave pattern is normal or curly, the wavelength has an average value of ~1.1–1.3 μm, and the amplitude is 0.26–0.35 μm. The amplitude of the waves in *B. fluminensis* and *B. dextrii* strains is usually somewhat larger (0.26–0.35 μm) than in strains of *B. indica* (0.26 μm) (Thompson and Skerman, 1979).

Colonial and cultural characteristics On agar media, especially under N₂-fixing conditions, *Beijerinckia* strains may produce giant colonies containing copious amounts of exopolysaccharide. This slime is often extremely tough, tenacious, or elastic, which makes it difficult to remove part of a colony with a loop. In *B. mobilis* and *B. fluminensis* the exopolysaccharide has a more granular consistency (Becking, 1984a).

On nitrogen-free mineral agar medium¹, *Beijerinckia* species exhibit various kinds of colonial characteristics and pigmentation, and in liquid cultures they show differences in viscosity and pellicle formation; see the individual species descriptions for details.

1. N-free mineral medium, g/l: glucose, 20.0; K₂HPO₄, 0.8; KH₂PO₄, 0.2; MgSO₄·7H₂O, 0.5; FeCl₃·6H₂O, 0.025 or 0.05; Na₂MoO₄·2H₂O, 0.005; CaCl₂, 0.05; and agar, 15.0. The pH is adjusted to 6.9. The CaCl₂ may be omitted to obtain a calcium-free medium.

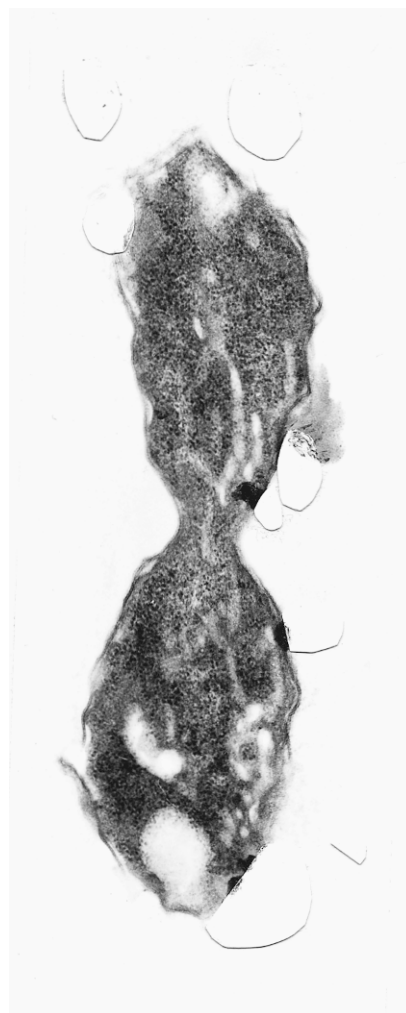


FIGURE BXII.α.159. Electron micrograph of a *Beijerinckia* cell in the process of division. The constriction in the middle of the cell is clearly visible and the two terminal lipid bodies of the original cell can be seen (× 33,000).

The temperature range for growth of *Beijerinckia* species is from 10 to 35°C. Cells are resistant to freezing; no reduction of viability occurs when stored for 3–4 months at –4°C (Becking, 1961).

Metabolism and metabolic pathways Carbon sources utilized include many sugars, organic alcohols, and organic acids (see Table BXII.α.141 and Table BXII.α.142). Since the compilation of strain characteristics by Thompson and Skerman (1979) and Becking (1984a), few reports of new features have appeared. One report describes the ability of *B. mobilis* to utilize several aromatic compounds as carbon sources and as energy sources for nitrogen fixation (Chen et al., 1993); these include benzoate, catechol, 4-hydroxybenzoate, naphthalene, protocatechuate, and 4-toluate. Most *Beijerinckia* species hydrolyze starch.

In alkaline, nitrogen-free, glucose minimal medium, *Beijerinckia* strains decrease the pH to 4.0–5.0. The acids produced are mainly acetic and a small amount of lactic (Kauffmann and Toussaint, 1951; Becking, 1961).

As with other N₂ fixers, *Beijerinckia* species require molybdenum for optimal growth and N₂ fixation. The molybdenum requirement—4.0–35.0 mg/l (Becking, 1962)—is notably higher

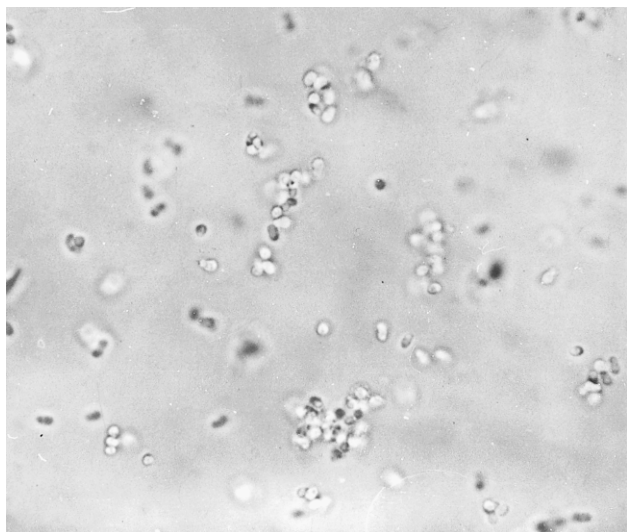


FIGURE BXII.α.160. *Beijerinckia mobilis* from an aged culture. The individual cells often lack the characteristic polar lipoid bodies and are more rounded in form, resembling certain *Azotobacter* species ($\times 1000$).

than that of *Azotobacter* and *Azomonas*. Moreover, unlike *Azotobacter*, the molybdenum cannot be replaced by vanadium (Becking, 1962). This finding suggests that alternative nitrogenases are not present in *Beijerinckia*, at least not the nitrogenase that contains vanadium (nitrogenase-2) (for further explanation, see the genus *Azotobacter*, this volume). Growth of *Beijerinckia* species under N_2 -fixing conditions does not require calcium, in contrast to most (but not all) *Azotobacter* species; indeed, $CaCO_3$ is even slightly inhibitory because it tends to prolong the lag phase of growth. The efficiency of N_2 fixation in *Beijerinckia* strains varies from 6 to 17 mg N_2 per g glucose consumed and is inversely proportional to growth (Becking, 1984a); the efficiency is greater at lower carbohydrate and/or oxygen supply levels (Spiff and Odu, 1973; Becking, 1978). The exocellular polysaccharide produced in copious amounts by species of *Beijerinckia* may protect nitrogenase from oxygen damage, as was shown for *B. dextrii* subsp. *venezuelae* (Barbosa and Altherthum, 1992). Removal of the exopolysaccharide resulted in a drastic loss of nitrogenase activity.

Strains of *Beijerinckia indica* and *Beijerinckia mobilis* have been reported to contain large amounts of triterpenoids of the hopane series. In the former species, both bacteriohopanetetrol and aminobacteriohopanetriol were present, whereas in the latter, amino-bacteriohopanetriol was the only C35 hopanoid (Vilcheze et al., 1994). Whether this difference represents a distinguishing feature for all, or several, members of each species was not indicated.

Occurrence and transfer of plasmids No studies involving isolation of mutants or genetic analysis of metabolism have been reported. The broad-host-range IncP plasmid RP4 could be mobilized into *B. indica* by conjugation and stably maintained (Naik et al., 1994). A tributyltin-sensitive strain, *Beijerinckia* sp. MC-27, isolated from freshwater sediment became resistant to this compound after transfer of a plasmid associated with chromium resistance from *Pseudomonas aeruginosa* PAO1 (Miller et al., 1995). The presence of indigenous plasmids in species of *Beijerinckia* was reported (Murai et al., 1990) but no function could be assigned.

Ecology *Beijerinckia* strains were originally isolated from a quartzite soil (pH 4.5) of Malaysia (Altson, 1936) and later from acid soils of Dacca, Bangladesh (pH 4.9), and Insein in Burma (pH 5.2) by Starkey and De (1939). They were later observed to be widely distributed in acidic tropical soils of Africa, Southeast Asia, and South America (Kluyver and Becking, 1955; Becking, 1961). *B. indica* is the most commonly encountered species and has been isolated from tropical soils of all continents and sometimes from nontropical regions. *Beijerinckia* strains can also be recovered from waterlogged soils of wet rice fields (Becking 1961, 1978). In a survey of 392 soils of worldwide distribution, *Beijerinckia* strains were found infrequently in some temperate and subtropical soils (Becking, 1959, 1961; see also the review by Becking, 1981). Nevertheless, their occurrence in temperate habitats might be more widespread than originally thought, based on reports of *Beijerinckia* spp. being isolated from European white fir (Streichan and Schink, 1986). Moreover, acidophilic, methanotrophic organisms with a soluble methane monooxygenase were isolated from three boreal forests in acidic northern wetlands of West Siberia and European north Russia (Dedysh et al., 1998a), and analysis of 16S rDNA sequence from these organisms placed them close to *Beijerinckia indica*. However, more recent studies show that these organisms—unlike *Beijerinckia* species—are unable to grow on sugars and other multicarbon compounds. In addition, *Beijerinckia* cannot grow on methane, and genes coding for methane monooxygenase have not been detected in *B. indica* (Dedysh, personal communication). These bacteria are now named *Methylocella palustris* (Dedysh et al., 2000).

B. mobilis occurs mainly in very acid soils (pH 4.0–4.5) of tropical Southeast Asia, Africa, and South America, and this is in accordance with its ability to fix nitrogen optimally at pH 3.9 (Becking, 1961). This high degree of acid tolerance might be useful for the specific enrichment and isolation of this species. *B. fluminensis* was originally isolated from a “Baixada Fluminensis” (pH 4.2–5.2) of Rio de Janeiro and from some other Brazilian soils (Döbereiner and Ruschel, 1958). It was also isolated from several African and Asian soils (Becking, 1961). *B. dextrii* was originally isolated from an Australian soil (Tchan, 1957) and then found in several South American and Asian locations, as well as in further locations in Australia (Thompson and Skerman, 1979).

In addition to soil or water habitats, *Beijerinckia* species are also found in plant rhizosphere and phyllosphere habitats. Associations with roots of sugarcane in Brazil and with a salt-tolerant grass *Leptochloa fusca* in Pakistan have been reported (Zafar et al., 1987; Baldani et al., 1997). *Beijerinckia* species have also been isolated from the leaves of plants such as coffee, cocoa, and cotton (Ruinen, 1956, 1961; Murty, 1984). There is no evidence that nitrogen fixation by *Beijerinckia* species provides an amount of fixed nitrogen sufficient to benefit plant growth.

ENRICHMENT AND ISOLATION PROCEDURES

An acidic, nitrogen-free medium² can be used for selective isolation of *Beijerinckia* from soil. The low pH of this medium favors development of beijerinckias, which are acid tolerant, and inhibits growth of other organisms, especially *Azotobacter* species. The requirement for trace elements (iron, molybdenum) in this medium is provided by the soil used as the inoculum. The medium is poured as thin layers (2–3 mm deep) into Petri dishes to allow good aeration. This partially inhibits the development

2. Enrichment medium (g/l of distilled water): glucose, 20.0; KH_2PO_4 , 1.0; and $MgSO_4 \cdot 7H_2O$, 0.5. The pH is adjusted to 5.0.

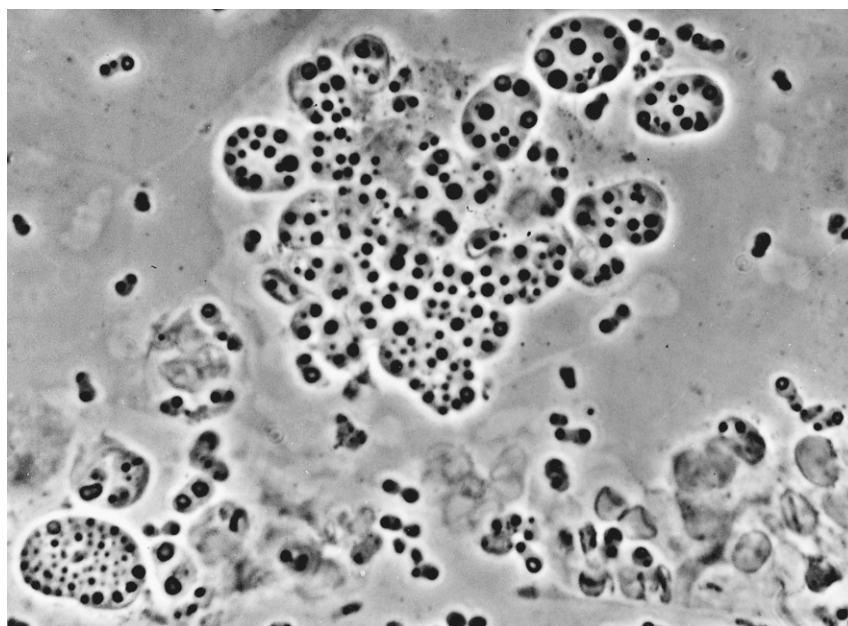


FIGURE BXII.α.161. *Beijerinckia fluminensis* cultured in nitrogen-free glucose mineral agar (pH 5.0), showing distinct capsule formation. The capsules enclose a large number of individual cells. Living preparation, phase-contrast microscopy ($\times 1500$).

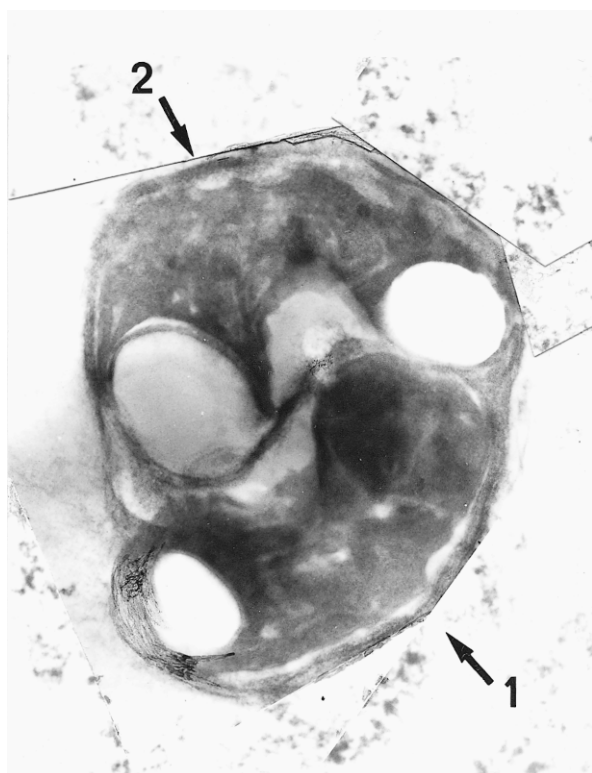


FIGURE BXII.α.162. Electron micrograph of a capsule of *Beijerinckia mobilis* containing two cells (see arrows 1 and 2). The terminal lipoid bodies of each cell are visible and also the distinct capsular wall (see arrow 1) ($\times 33,000$).

of anaerobic or facultative N_2 fixers. Approximately 0.5 g of soil, 10 ml of water sample, or detached leaves can be used as the inoculum. The enrichment cultures are incubated at 30°C for 2

or more weeks (*Beijerinckia* strains grow slowly). The entire culture may eventually change into a viscous mass due to slime production. The cultures are examined microscopically at various times for the presence of characteristic *Beijerinckia*-like cells. When such cells occur, the enrichment culture is plated onto a nitrogen-free, mineral agar medium (see Further Descriptive Information). Although an acidic agar medium can be used for isolation, it is not recommended because the agar may be hydrolyzed.

The sieved-soil plate method of Winogradsky (1932), using nitrogen-free mineral agar (pH 4.5–5.0) with glucose or sucrose (10 or 20 g/l), can also be applied. *Beijerinckia* colonies develop after 2–3 weeks around the soil particles on the plates. The type strain of *B. indica* was obtained by this method. In general, however, it is less satisfactory than the use of liquid enrichment media because purification is more difficult (the slime is more tenacious) when one starts with solid media. If the plating method is chosen, a drop inoculation is recommended rather than spreading, because of the higher number of colonies obtained with the former approach (Barbosa et al., 1995).

On agar media, *Beijerinckia* species form characteristic, highly raised, glistening colonies containing a tough, elastic slime. For further purification, a similar medium is used, but it is made neutral or alkaline by the addition of $CaCO_3$ ³. This is because the slime is more soluble under alkaline conditions, and it is easier to suspend the cells in sterile tap water or liquid medium for further streaking. On the alkaline agar medium, *B. indica* strains usually form highly raised colonies, whereas *B. mobilis* colonies are flatter and produce a uniform reddish brown or amber-brown color on aging.

3. N-free mineral agar medium with $CaCO_3$; composition is similar to that given in Further Descriptive Information, but the $CaCl_2$ is replaced by $CaCO_3$ (10–20 g/l).

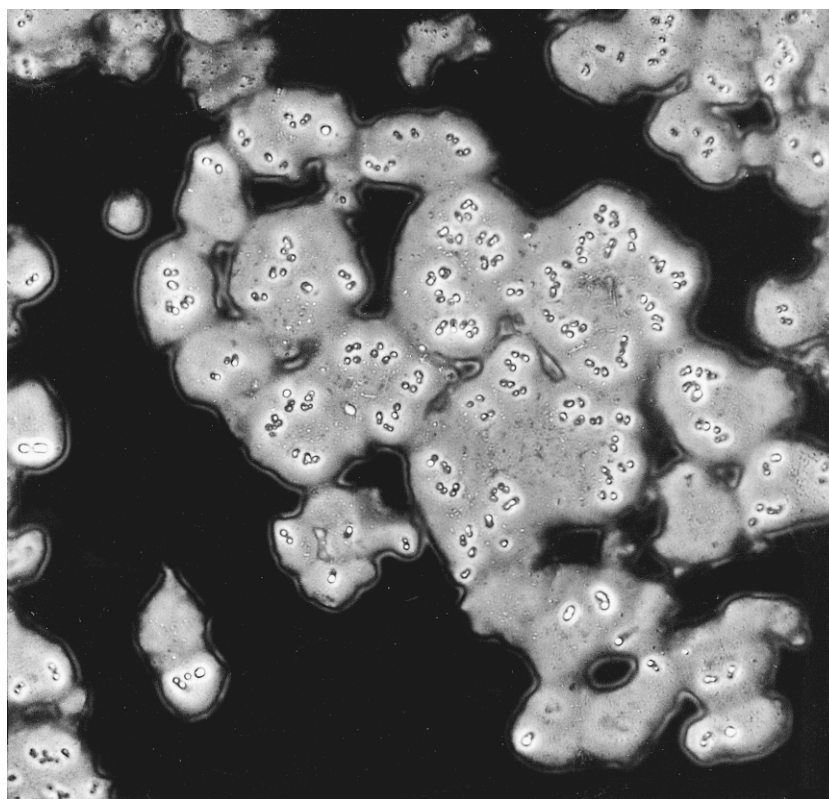


FIGURE BXII.α.163. Cells of *Beijerinckia indica* suspended in India ink, showing the polysaccharide formation around the cells. Living preparation, phase-contrast microscopy. ($\times 1350$)

TABLE BXII.α.141. Differential characteristics of the species of the genus *Beijerinckia* and the genus *Derrxia*^a

Characteristics	<i>B. indica</i>	<i>B. derxii</i>	<i>B. fluminensis</i>	<i>B. mobilis</i>	<i>Derrxia gummosa</i>
Water-soluble, green fluorescent pigment	—	+	—	—	—
Colony color after aging	P	B	P	AB	Br
Motility	[—] ^b	—	[—] ^b	+	+
Resistant to 1% peptone	d	—	—	d	+
Starch hydrolyzed	—	d	—	—	—
Growth on asparagine as C and N source	—	—	—	d	+
Urea hydrolyzed	+	+	—	+	+
H ₂ S production from cysteine	—	—	+	—	—
Tween 20 hydrolyzed	—	—	—	—	+
Indole produced	—	—	—	—	+
Antagonism to Gram positive organisms	—	d	—	—	+

^aFor symbols, see standard definitions; P, pink; B, buff; AB, amber-brown; Br, brown. For additional distinguishing characteristics, see Table BXII.α.142, utilization of carbon compounds.

^bIf positive, motility occurs mostly in young stages and the cells are usually only weakly motile.

Specific enrichment procedures No specific procedure is known to select for a particular *Beijerinckia* species, and all existing strains are random isolates obtained from soil using one of the general procedures outlined above. In general, carbon source utilization is not distinctive for particular species, although certain substrates might be useful for enrichment of one or two particular species because of their preferential use: e.g., benzoate for enrichment of *B. mobilis* and mannose for *B. indica* or *B. fluminensis* (see Table BXII.α.142). Thompson and Skerman (1979) suggested that certain substrates or inhibitors might be useful for enrichment of various species, although this has not yet been experimentally tested. From the properties of the strains

studied, the following compounds have potential value for enrichment or selection:

1. *B. indica*: L-arabinose, D-mannose, glycerol, caprylate, and *trans*-aconitate. Glycerol might be useful for *B. indica*, and for *Derrxia gummosa* and some *B. fluminensis* strains. Nitrotriacetate might also be useful for *B. indica*.
2. *B. mobilis*: pentan-2-ol, 1,3-butylene glycol, asparagine, and *n*-valerate might be useful. Phenol (0.05%) might be used to inhibit the growth of other *Beijerinckia* species.
3. *B. indica* and *B. mobilis*: propan-1-ol, butan-1-ol, or 1,3-propylene glycol for enrichment.

4. *B. indica* subsp. *lacticogenes* and *B. mobilis*: caproate, *p*-hydroxybenzoate, or phenol for enrichment.
5. *B. derxii* and *B. fluminensis*: α -methyl-D-glucoside, maltose, melibiose, or melezitose for enrichment. Where both species are present, *B. derxii* would likely outgrow the extremely slow-growing *B. fluminensis*.
6. *B. derxii* subsp. *venezuelae*: L-arabitol for enrichment.

Because the colony morphology and chromogenesis of growth on solid media differs among the various *Beijerinckia* species, primary selection of colonies from the enrichment cultures is done mainly on the basis of these colonial characteristics. A more precise identification is performed later using additional differential characteristics.

MAINTENANCE PROCEDURES

Beijerinckia strains can be lyophilized in skim milk or dextran-sodium glutamate solution on filter paper and stored in the dark at room temperature (Becking, 1984a).

Storage has also been achieved on the usual agar media in tubes plugged with sterile rubber seals, with storage in the dark at room temperature (Antheunisse, 1972, 1973); after 10 years, 33% of the cultures retained viability. *Beijerinckia* cultures stored under a seal of sterile liquid paraffin or mineral oil generally survive for at least 3–5 years (Becking, 1984a).

Strains may also be preserved indefinitely in liquid nitrogen. At the type culture collection in Delft, The Netherlands, DMSO (10%, v/v) is added to cultures in the log phase or end of log phase, and the cultures are frozen as rapidly as possible in liquid nitrogen. For recovery, the vials are thawed rapidly in a waterbath at 37°C (Becking, 1984a).

Cryopreservation in 10% glycerol was also successful (Thompson, 1987). See the genus *Azotobacter* for details.

DIFFERENTIATION OF THE GENUS *BEIJERINCKIA* FROM OTHER GENERA

Beijerinckia strains can be distinguished from other aerobic N₂ fixers by their great acid tolerance (which allows them to grow well at pH 4.0 or 5.0), by their failure to form a pellicle on the surface of liquid media, and by their ability to make a liquid medium viscous by slime production (Fig. BXII.α.164). Moreover, on solid media they produce characteristic large, slimy colonies having a tough, tenacious, and sometimes elastic slime.

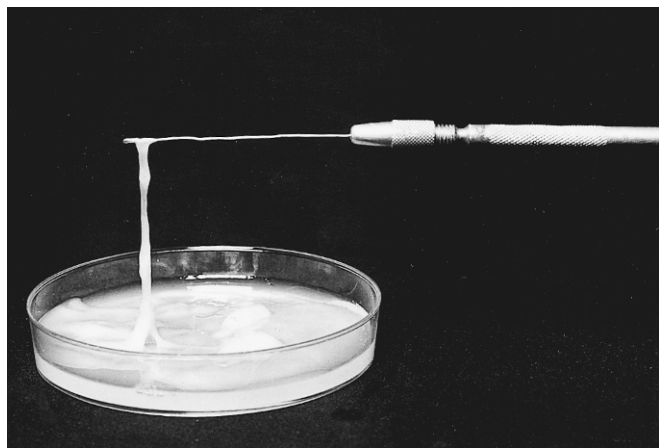


FIGURE BXII.α.164. Enrichment culture of *Beijerinckia* inoculated with tropical soil, demonstrating the highly viscous consistency of the medium after 3 weeks ($\times 0.8$).

Because of this exopolysaccharide production, it is often difficult to subculture portions of a colony for purification. For other features distinguishing *Beijerinckia* strains from *Derxia**, the most closely related genus, see Tables BXII.α.141 and BXII.α.142.

Beijerinckia cells can be distinguished from those of *Azotobacter* and *Azomonas* by their generally smaller size, by their more rod-shaped or sometimes pear- or dumbbell-shaped appearance, and by the characteristic presence of a lipid body at each pole (Fig. BXII.α.156). Many strains of *Beijerinckia* utilize nitrate poorly or not at all and, in this respect, differ from strains of *Azotobacter*.

Although both *Beijerinckia* and *Derxia* produce slimy colonies on agar and viscosity in broth, *Beijerinckia* strains can be distinguished by (a) failure to produce dark mahogany-brown colonies with aging, (b) cells that contain bipolar lipid bodies rather than numerous lipid bodies throughout the whole cell, (c) failure to form a pellicle at the surface of liquid media, and (d) a positive catalase reaction. In addition, a number of C sources should distinguish the two genera; for instance, *Beijerinckia* species, but not *Derxia*, can utilize sorbose and raffinose, and *Derxia*, but not *Beijerinckia*, utilizes aspartate, glutamate, or ethylamine (Table BXII.α.142).

TAXONOMIC COMMENTS

When numerical analysis methods are applied to species of *Beijerinckia*, *Azotobacter*, and *Azomonas*, the *Beijerinckia* species fuse into a single, apparently coherent group (Thompson and Skerman, 1979). Using a wide range of attributes, and considering all strains of named and unnamed *Beijerinckia* species, numerical analysis supports the concept of a separate genus for these bacteria. In addition, the experiments reported by De Smedt et al. (1980), in which ¹⁴C-labeled rRNA from *Beijerinckia indica* was hybridized with filter-fixed DNA from a wide variety of Gram-negative bacteria, indicated the genus *Beijerinckia* to be a heterogeneous but coherent group. From rRNA cistron similarities, it was concluded that *Beijerinckia* and *Azotobacter*/*Azomonas* belong to different classes. Analysis of the 16S rDNA sequence of *Beijerinckia indica* places this genus within the phylum *Proteobacteria*. The family *Beijerinckiaceae*, including the genera *Beijerinckia*, *Derxia*, and *Chelatococcus*, is in the class *Alphaproteobacteria* and the order *Rhizobiales*. This family is most closely related to the families *Methylocystaceae* and *Bradyrhizobiaceae*.

Thompson and Skerman (1979) used many phenotypic characters of *Beijerinckia* strains for numerical analysis, yielding a hierarchical classification. These authors confirmed the presence of four species, in two main groups within the genus. Group 1294 (*B. fluminensis* + *B. derxii*) fuses with group 1289 (*B. indica* subsp. *indica* + *B. indica* subsp. *lacticogenes* + *B. mobilis*) to produce the group 1297. Group 1289 strains generally differed from group 1294 strains in having thinner cells and using caprylate, propan-1-ol, 1,3-propylene glycol, *trans*-aconitate, nitrilotriacetate, and L-arabinose, but not maltose and α -methyl-D-glycoside, as sole carbon sources. Moreover, strains of group 1289 produced acid from L-arabinose and glycerol and were resistant to 0.5% NaCl, 0.05% phenol, and 1.0% sodium benzoate. One widely studied member of the genus, *Beijerinckia* sp. strain B1, was reclassified as a strain of *Sphingomonas yanoikuyae* on the basis of 16S rDNA analysis (Khan et al., 1996a).

*Editorial Note: Because of the 16S rDNA placement, the genus *Derxia* has been moved to the family *Alcaligenaceae* and is no longer listed in *Beijerinckiaceae*.

TABLE BXII.α.142. Utilization of carbon compounds by *Beijerinckia* and *Derxia* species^a

Carbon compounds utilized ^{b,c}	<i>B. indica</i> (14) ^d	<i>B. derxii</i> (21)	<i>B. fluminensis</i> (10)	<i>B. mobilis</i> (2)	<i>Derxia gummosa</i> (6)
Arabinose	+	—	d	+	—
Galactose	+	+	+	—	—
Fructose	+	+	+	—	+
Melibiose	d	d	+	—	—
Maltose	—	+	d	—	—
Mannose	+	—	d	—	—
Sorbose	+	d	+	+	—
Raffinose	+	+	d	d	—
Xylose	—	—	+	—	—
Butanol	+	—	d	+	+
Propanol	+	—	—	+	+
Glycerol	+	—	+	+	+
Sorbitol	+	+	+	—	d
Mannitol	+	+	d	d	d
Acetate	d	+	—	+	+
Citrate	+	d	—	d	+
Oxaloacetate	+	d	—	—	+
Fumarate	+	d	d	d	+
Malate	+	d	—	d	—
Malonate	—	—	—	—	+
Glycolate	+	+	—	+	+
Benzoate	—	—	—	+	—
L-Ascorbate	+	d	+	—	—
Aspartate	—	—	—	—	+
Glutamate	—	—	—	—	+
Ethylamine	—	—	—	—	+

^aFor symbols, see standard definitions.^bData represent a merger of information in Thompson and Skerman (1979) and Becking (1984a) in the 1st edition of *Bergey's Manual of Systematic Bacteriology*. Not all compounds tested by Thompson and Skerman (1979) are included.^cAll species can utilize glucose, sucrose, lactate, pyruvate, succinate, gluconate, and fumarate. All species fail to utilize ribose, fucose, cellobiose, lactose, trehalose, glutarate, and oxoglutarate.^dNumbers in parentheses represent the number of strains tested.**ACKNOWLEDGMENTS**

The description of the genus as given by J.-H. Becking in the 1st edition of the *Manual* remains largely unchanged; information has been reorganized, reevaluated, and updated.

FURTHER READING

Becking, J.H. 1992. The Genus *Beijerinckia*. In Balows (Editor), *The Prokaryotes. A Handbook on the Biology Of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd Ed., Vol. 3, Springer-Verlag, New York. pp. 2254–2267.

List of species of the genus *Beijerinckia*

1. ***Beijerinckia indica*** (Starkey and De 1939) Derx 1950a, 146^{AL} (*Azotobacter indicum* (sic) Starkey and De 1939, 337.) *in' di.ca*. L. fem. adj. *indica* of India.

The characteristics are as described for the genus and as given in Tables BXII.α.141 and BXII.α.142, with the following additional information. Straight or slightly curved rods 0.5–1.2 × 1.6–3.0 μm. PHB granules persist in aged cultures. No resting stages occur; neither cyst nor spore formation is observed.

Agar colonies are raised. At first, they are semitransparent but soon become uniformly turbid or opaque white. On aging, the colonies develop a light reddish pink, cinnamon, or fawn color on neutral or alkaline media; on acid media, they remain colorless. On acid media, the slime is more tenacious, tough, and elastic than on alkaline media. Giant colonies may develop, first with a smooth surface (Fig. BXII.α.165), but later with a folded or wrinkled surface (Fig. BXII.α.166).

Liquid media become viscous as cell density increases.

Color may be produced, but is less prominent than on agar.

Grows between pH 3.0 and 10.0 (optimum is 4.0–10.0). Temperature range for growth, 10–35°C; no growth at 37°C.

Growth on, and utilization of, nitrate is poor, and N₂ is fixed in preference to utilization of nitrate in the medium (Becking, 1962). Weak growth occurs on malt agar, no growth in plain broth or peptone agar.

Widely distributed in acid tropical soils.

The mol% G + C of the DNA is: 54.7–58.5 (*T_m*) (De Ley and Park, 1966; De Smedt et al., 1980).

Type strain: Delft E.II.12.1.1, ATCC 9039, DSM 1715, NCIB 8712, WR-119.

GenBank accession number (16S rRNA): M59060.

Derx (1950a) described "*B. indica* biovar *alba*", which was distinguished by its lack of pigmentation on aging; however, under extreme (alkaline) conditions it produced a pink pigment. In the hierarchical classification of Thompson and Skerman (1979), one co-type (strain WR-236) of *B. indica* biovar *alba* was placed in *B. indica* subsp. *lacti-*

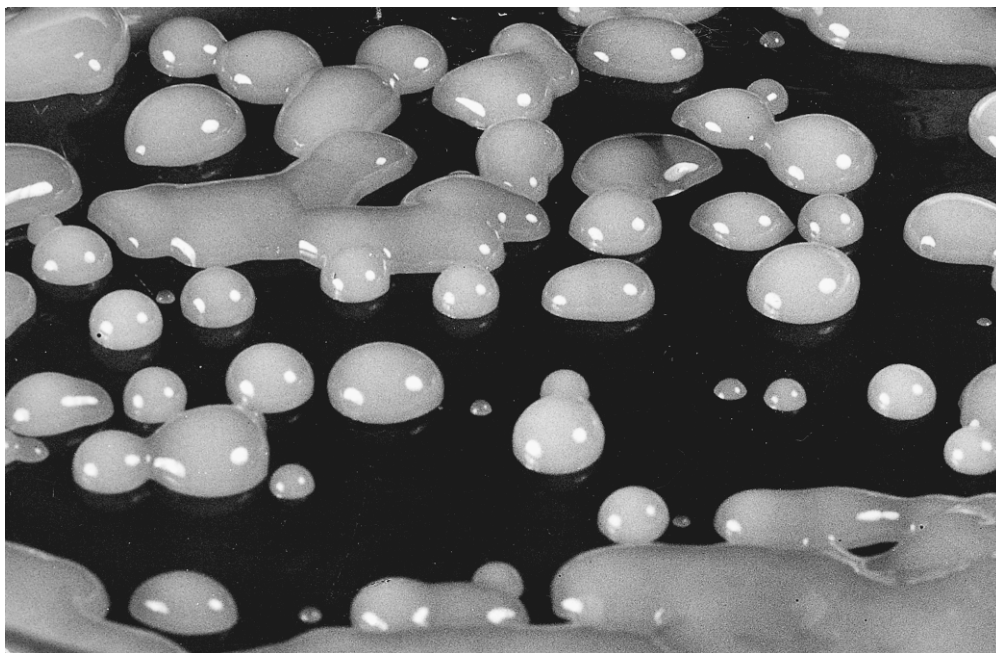


FIGURE BXII.α.165. Typical colony type of *Beijerinckia indica* on nitrogen-free glucose mineral agar. The colonies are highly raised and have a very tough, elastic slime. In young cultures these colonies are colorless and transparent ($\times 2$).



FIGURE BXII.α.166. Typical colony of an aged culture of *Beijerinckia indica* on nitrogen-free glucose mineral agar. The colonies increase greatly in size due to copious slime production. The colonies become massive and opaque, with a plicate surface. In this stage they often attain a light reddish, pink, or cinnamon color, especially on neutral or alkaline media ($\times 2$).

cogenes (group 1270) and the other (strain WR-235) was placed in *B. derxii* subsp. *venezuelae* (group 1271).

- a. ***Beijerinckia indica* subsp. *indica*** (Starkey and De 1939) Derx 1950a, 146^{AL} (*Azotobacter indicum* (sic) Starkey and De 1939, 337.)

Thompson and Skerman (1979) distinguished *B. indica* subsp. *indica* from subsp. *lacticogenes* by differences in organic carbon utilization, nitrate reduction, and resistance to peptone-nitrogen. Based on their study of nine strains of subsp. *indica* and four or five strains of

subsp. *lactico*genes, the only absolute character that differentiated the two subspecies was the failure of subsp. *indica* to utilize *p*-hydroxybenzoate as a sole carbon source. Nitrate was reduced to nitrite by eight of nine strains of subsp. *indica* but not by four of four strains of subsp. *lactico*genes. The differences in utilization of sole carbon sources by subsp. *indica* vs. subsp. *lactico*genes were as follows: propan-2-ol, 7/9 vs. 5/5; butan-2-ol, 0/9 vs. 3/5; D-arabitol, 4/9 vs. 1/5; phenol, 0/9 vs. 4/5; caproate, 0/9 vs. 4/5; adipate, pimelate, suberate, azelate, and sebacate, 0/9 vs. 1/5 or 1/4; α -oxybutyrate, 0/9 vs. 1/5; fumarate, DL-malate, tartrate (D, L, and *meso*), oxaloacetate, mucate, and *trans*-aconitate, 9/9 vs. 3–4/5. None of the strains of subsp. *indica* lacked flagella, whereas half the strains of subsp. *lactico*genes did lack flagella.

The mol% G + C of the DNA is: 54.7–58.5 (T_m) (De Ley and Park, 1966; De Smedt et al., 1980).

Type strain: Delft E.II.12.1.1, ATCC 9039, DSM 1715, NCIB 8712, WR-119.

GenBank accession number (16S rRNA): M59060.

- b. ***Beijerinckia indica* subsp. *lactico*genes** (Kauffmann and Toussaint 1951) Thompson and Skerman 1981, 215^{VP} (Effective publication: Thompson and Skerman 1979, 332) (*Azotobacter lactico*genes Kauffmann and Toussaint 1951, 710)

lac.ti.co'ge.nes. M.L. n. *acidum lacticum* lactic acid; Gr. v. *genmaio* to produce; M.L. adj. *lactico*genes lactic acid producing (which is an error: acetic acid is produced).

This subspecies is distinguished from *B. indica* subsp. *indica* by the characteristics described above, and by the consistency of the colonies (less elastic and rubbery (cartilaginous) and more butyrous and brittle than those of subsp. *indica*). Moreover, subsp. *lactico*genes is somewhat less resistant to diamond fuchsin, brilliant green, sodium fluoride, and streptomycin. In contrast to subsp. *indica*, four of five strains of subsp. *lactico*genes grown on agar containing *p*-hydroxybenzoate could metabolize protocatechuate via the *ortho*-cleavage pathway (Thompson and Skerman, 1979).

The mol% G + C of the DNA is: unknown.

Type strain: WR-119, ATCC 19361.

2. ***Beijerinckia derxii*** Tchan 1957, 315^{AL}

derx' i. i. M.L. gen. n. *derxii* of Derx; named after H.G. Derx, the Dutch microbiologist (1894–1953).

The characteristics are as described for the genus and as given in Tables BXII.α.141 and BXII.α.142, with the following additional information. Single straight or curved rods, or rods with clavate extremities, 1.5–2.0 × 3.5–4.5 μm. Polar lipid bodies are very large and conspicuous. No cyst or capsule formation occurs. Nonmotile.

Colonies are highly raised, slimy, and smooth. The chemical composition of the slime has not yet been examined. Colonies are at first semitransparent or opaque white, but after 2–3 weeks, a yellow-green, water-soluble, fluorescent pigment is produced, particularly on iron-deficient media. When the pigment first appears it remains within the colony, but later it diffuses into the agar medium. Under certain conditions, pigment production on agar media may be very poor or absent.

Liquid cultures become uniformly turbid and pigment production is usually less than on solid media.

Growth occurs between pH 4.0 and 9.0 (optimum, 6.0–7.0). There is no growth at pH 3.0 or 11.0. Temperature range for growth, 10–35°C; no growth at 37°C.

Isolated from soils from Queensland, Northern Australia, and neutral and alkaline soils of South Africa.

The mol% G + C of the DNA is: 59.1 ± 1.6 (T_m) (De Ley and Park, 1966).

Type strain: Q13 of Tchan, ATCC 49361, DSM 2328, UQM 1968.

a. ***Beijerinckia derxii* subsp. *derxii*** Tchan 1957, 315^{AL}

In the hierarchical classification of Thompson and Skerman (1979), the strains within the species *B. derxii* can be divided into two main groups: group 1263 and group 1271. Group 1263 contains a co-type of *B. derxii* Tchan 1957 and three co-type strains of "*Beijerinckia congensis*" Hilger 1965, as well as three Australian isolates. This group was named by Thompson and Skerman (1979) as *B. derxii* subsp. *derxii*. Differences between this subspecies and *B. derxii* subsp. *venezuelae* (group 1271) are not markedly consistent, apart from utilization of nitrate, as outlined below in the description of the subsp. *venezuelae*.

The mol% G + C of the DNA is: not available.

Type strain: Q13 of Tchan, ATCC 49361, DSM 2328, UQM 1968.

b. ***Beijerinckia derxii* subsp. *venezuelae*** (Materassi, Florenzano, Balloni and Favilli 1966) Thompson and Skerman 1981, 215^{VP} (Effective publication: Thompson and Skerman 1979, 343) (*Beijerinckia venezuelae* Materassi, Florenzano, Balloni and Favilli 1966, 210.)

ven.e.zue' lae. M.L. gen. n. *venezuelae* of Venezuela, South America.

In the hierarchical classification of Thompson and Skerman (1979), group 1271 is classified as *B. derxii* subsp. *venezuelae*. This group consists of six co-type strains of "*B. venezuelae*" Materassi et al. 1966, one co-type of "*B. indica* biovar *alba*" Derx 1950a, and three Australian isolates.

Differences between group 1263 (subsp. *derxii*) and group 1271 (subsp. *venezuelae*) are not markedly consistent. In general, strains of group 1271 are distinguished by not utilizing nitrate as a sole source of nitrogen, being nonmotile, not hydrolyzing glycogen, growing over a slightly wider pH range (not more than 0.5 pH unit at each end of the range), and by differences in utilization of several organic compounds as sole sources of carbon. Of 14 strains of subsp. *venezuelae* and 7 strains of subsp. *derxii* tested by Thompson and Skerman (1979), the utilization of these substrates was as follows: melibiose, 5/14 vs. 7/7; propan-2-ol, 6/14 vs. 0/7; L-arabitol, 6/14 vs. 0/7; propionate, 7/14 vs. 0/7; fumarate, 8/14 vs. 1/7; and L-ascorbate, 6/14 vs. 5/7. Eleven of 14 strains of subsp. *venezuelae* used nitrate as a nitrogen source, whereas only 1 of 7 strains of subsp. *derxii* could do so.

The mol% G + C of the DNA is: not available.

Type strain: 2 of Materassi, DSM 2329, WR-222.

Of the six strains of subsp. *venezuelae* provided by G. Florenzano (i.e., strains WR-221 to WR-226), it is not

clear how many independent isolates are represented (Becking, 1984a).

3. **Beijerinckia fluminensis** Döbereiner and Ruschel 1958, 269^{AL}

flu.mi.nen'sis. M.L. adj. *fluminensis* named after the locality "Baixada Fluminense", State Rio de Janeiro, Brazil, from which soil it was first isolated.

The characteristics are as described for the genus and as given in Tables BXII.α.141 and BXII.α.142, with the following additional information. Straight or slightly curved rods, $1.0\text{--}1.5 \times 3.0\text{--}3.5 \mu\text{m}$. Older cultures show characteristic large capsules enclosing 2–10 or more individual cells. Division of cells within the capsules has been observed. Motility is slow or absent, especially in older cells.

Colonies are typically small and granular, moderately raised, with an irregular rough surface (Fig. BXII.α.167). The slime is not liquid, tenacious, or elastic, but more granular and stiff; its chemical composition has not yet been examined. Colonies are at first opaque white, becoming pink, reddish brown, or fulvous (like *B. indica*) after 1–2 weeks on neutral or alkaline media.

Slime production in liquid media is reduced. No pellicle or viscosity occurs, but a bluish white turbidity develops.

Grows between pH 3.5 and 9.2. Temperature range for growth, 10–35°C (optimum, 26–33°C); no growth at 37°C.

Found in acidic soils of South America, Africa, and Asia (China, Indonesia).

The mol% G + C of the DNA is: 56.2 ± 1.8 (T_m) (type strain) (De Ley and Park, 1966; De Smedt et al., 1980).

Type strain: CD10 of Döbereiner and Ruschel, DSM 2327, UQM 1685.

4. **Beijerinckia mobilis** Derx 1950b, 10^{AL} (*Beijerinckia mobile* (sic) Derx 1950b, 10.)

mo'bi.lis. L. fem. adj. *mobilis* movable, motile.

The characteristics are as described for the genus and as given in Tables BXII.α.141 and BXII.α.142, with the following additional information. Straight, curved, or pear-

shaped rods, $0.6\text{--}1.0 \times 1.6\text{--}3.0 \mu\text{m}$. Misshaped or forked cells sometimes occur. "Ascococcus"-like clusters of cells are often visible in older cultures. The typical polar lipid bodies may disappear in aging cells, and the cells are then more rounded and resemble *Azotobacter* cells (Fig. BXII.α.160). Motility is conspicuous.

Agar colonies are not as raised as those of *B. indica*, and slime production is less (Fig. BXII.α.168). The slime is neither elastic nor sticky; its chemical composition has not yet been examined. Older cultures on neutral or alkaline agar media show a typical dark amber or deep reddish brown color.

Broth cultures do not become viscous. There is a tendency to form a pellicle at the surface.

Grows between pH 3.0 and 10.0. Optimal growth and N₂ fixation occur at pH 4.0–5.0 and decrease sharply at the more alkaline values (Becking, 1961). Temperature range for growth, 10–35°C; no growth at 37°C.

All strains tested have grown well on nitrate or ammonium salts as the nitrogen source (in contrast to *B. indica*). Weak growth or no growth occurs on urea, glycine, glutamate, or tyrosine. All strains grow on leucine and casein agar. Moderate growth occurs on malt agar. The differences in levan production from sucrose observed by Derx (1950b) are variable and cannot be used for differentiation of this species.

Common in Indonesian (Java) soils; also isolated from soils of South America (Surinam) and tropical Africa.

The mol% G + C of the DNA is: 57.3 (T_m) (De Smedt et al., 1980).

Type strain: Delft E.III.12.2, ATCC 35011, DSM 2326, UQM 1969.

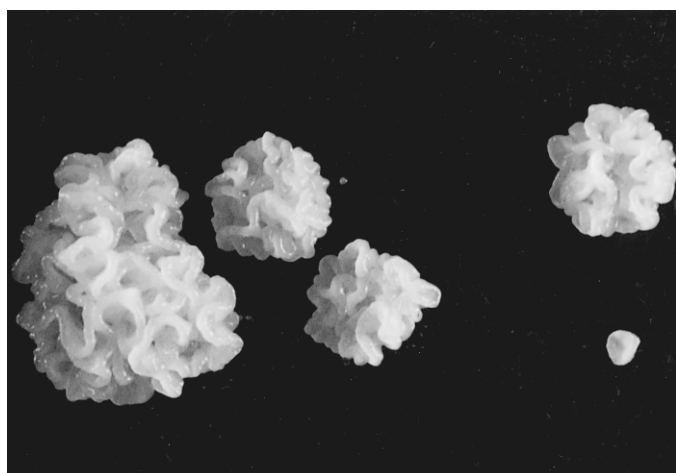


FIGURE BXII.α.167. Typical colonies of *Beijerinckia fluminensis* on nitrogen-free glucose mineral agar. This species forms rather small, raised colonies with a highly plicate surface. In this species the slime has a granular consistency ($\times 4$).

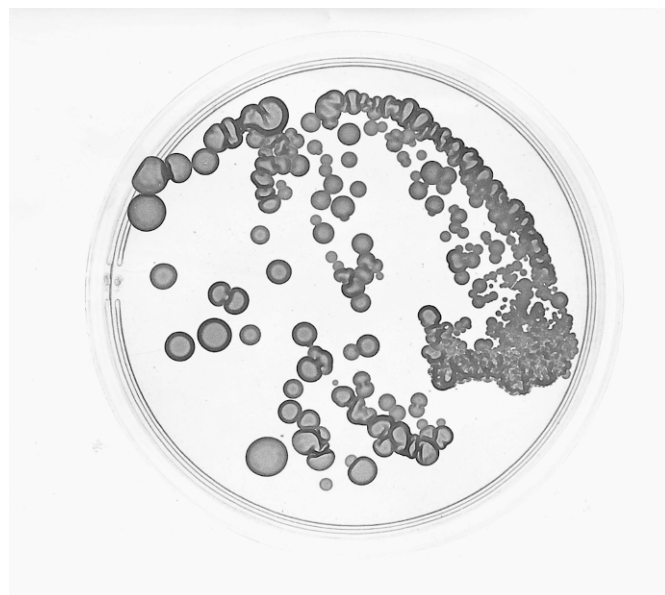


FIGURE BXII.α.168. Colonies of *Beijerinckia mobilis* on a nitrogen-free glucose mineral agar containing CaCl₂. On this transparent medium, the species forms only small raised colonies having a typical amber-brown color on aging ($\times 0.7$).

Genus II. Chelatococcus Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624^{VP}
(Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 109)

THOMAS W. EGLI AND GEORG AULING

Che.la'to.coc.cus. Gr. n. *chele* claw; M.L. v. *chelato* to form claw-like complexes with divalent cations, i.e., to chelate; Gr. n. *coccus* berry, used for a bacterium of roughly spherical shape; M.L. masc. n. *Chelatococcus* chelating coccus.

Short, stout, almost coccoid rods, 0.5–1 × 1–2 µm; resemble diplococci during exponential growth. Gram negative. **Nonmotile. Obligately aerobic.** Optimal growth temperature, 35–37°C; temperature range for growth, 4–41°C. Slow growth with all substrates tested so far ($\mu_{\max} < 0.2 \text{ h}^{-1}$). Poly- β -hydroxybutyrate is accumulated within the cells. **Utilize the metal-chelating aminopolycarboxylic acids nitrilotriacetic acid (NTA) and S,S-ethylenediaminedisuccinic acid as sole sources of carbon, energy, and nitrogen.** Generally, sugars are not utilized (except for glycerol). **Require one or more vitamins.** All strains are sensitive to β -lactam antibiotics but resistant to nalidixic acid. Ubiquinone Q-10 is present. The main polyamine present is *sym*-homospermidine; putrescine and spermidine occur as major polyamines, and spermine as a minor polyamine.

The mol% G + C of the DNA is: 63.

Type species: *Chelatococcus asaccharovorans* Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624 (Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 110.)

FURTHER DESCRIPTIVE INFORMATION

Morphological features The typical morphology of *Chelatococcus asaccharovorans* is shown in Fig. BXII.α.169a and b. Ultra-thin sections reveal a Gram-negative cell envelope with a cytoplasmic membrane, a murein layer 6–8 nm wide, an outer membrane, and an additional proteinaceous surface layer (Fig. BXII.α.169c, d). The murein layer is always in close contact with the outer membrane. The outer proteinaceous crystalline surface layer (S-layer) is approximately 15 nm wide. Its outer side appears smooth, whereas, depending on the plane of sectioning, the inward-facing side of the S-layer exhibits a regular arrangement of protrusions (Fig. BXII.α.169d). This S-layer is a rather brittle structure. Easy detachment of the S-layer may explain the patchy surface structure of deep-etched cells (Fig. BXII.α.169c). It is not yet known whether this is an intrinsic property of the S-layer or an artifact resulting from excessive mechanical stress during preparation of the cells for electron microscopy. Detached patches with a hexagonal arrangement are usually found in the culture medium (Wehrli and Egli, 1988). The repeating units are most probably hexamers, with a central hole and a center-to-center spacing of approximately 16 nm. Since smooth cell surfaces were occasionally observed in deep-etched samples of both strains, the possibility that the S-layer is covered by a further outer protein layer remains. Although such S-layers are common in both prokaryotic domains (*Bacteria* and *Archaea*), their value for taxonomic purposes has not yet been shown (Messner and Sleytr, 1992).

Colony morphology Colonies of *Chelatococcus* are similar in appearance to those of *Chelatobacter* (see Fig. BXII.α.132 in the genus *Chelatobacter*). After 4–5 d incubation at 30°C on complex medium (PCA, Plate Count Agar, Difco), *Chelatococcus asaccharovorans* strain TE1 develops round and smooth colonies with a diameter of 3–4 mm, which appear white-to-beige and mucoid. On selective NTA mineral medium (see below), a different colony morphology is observed with a stereo-microscope, 25-fold

magnification. Even well-developed colonies never exceed 0.5 mm in diameter. The smaller colony size on NTA agar (compared to growth on PCA) is probably due to a rise in pH resulting from the excretion of ammonia during growth with NTA as the only source of carbon, energy, and nitrogen. Colonies appear white (incident light) and translucent (transmitted light) when young (1–2 d). With prolonged incubation, the colonies are still white (incident light), but under the stereo-microscope (transmitted light) they appear brown and finally black, and their shape becomes “polygonal” (3–5 d) as shown in Fig. BXII.α.132. *Chelatococcus asaccharovorans* strain TE2 may be a nonmucoid mutant of strain TE1. Colonies are smaller (1 mm after 4–5 d) on both complex and mineral agar. They appear firm and raised on NTA agar.

Cultural characteristics The chemically-defined medium used for isolation, maintenance, and growth of *Chelatococcus asaccharovorans* strains in both batch and chemostat culture contains (g/l distilled water): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.41; KH_2PO_4 , 0.26; 1 ml of the trace element stock solution described by Pfennig et al. (1981) with NTA as the chelating agent (5.2 g/l), and 1 ml of a vitamin stock solution. The vitamin solution contains (per liter): pyridoxine-HCl, 100 mg; thiamine-HCl, riboflavin, nicotinic acid, D-calcium pantothenate, *p*-aminobenzoic acid, lipoic acid, nicotinamide and vitamin B₁₂, 50 mg each; biotin, 20 mg; and folic acid, 20 mg. This medium is supplemented with either NTA or other carbon and/or nitrogen sources (up to a maximum of 5 g/l carbon). For growth with carbon sources containing no nitrogen, the medium is supplemented with NH_4Cl (0.54 g/l). For isolation and growth on agar plates, this medium was supplemented with 1.5% agar plus NTA (0.5 g/l). Growth is dependent on addition of the above vitamin mixture (Egli et al., 1988) or yeast extract (0.1 g/l).

Growth characteristics Growth in batch culture with NTA as the only carbon/energy and nitrogen source results in the excretion of ammonia, which causes an increase in pH of the culture medium. Under such conditions, growth ceases at approximately pH 9 and after transfer into new medium, the cells exhibit extended lag times (often 5–10 d) until growth resumes. Growth with mixtures of utilizable carbon sources plus NTA results in simultaneous utilization of the two substrates, and in faster growth than with NTA alone. For example, during simultaneous utilization of acetate plus NTA a maximum specific growth rate of 0.13 h^{-1} was reported, compared to 0.07 h^{-1} during growth with NTA alone (see Table BXII.α.115 in the genus *Chelatobacter*).

Nutritional and physiological characteristics For the nutritional and physiological characteristics of *Chelatococcus asaccharovorans* strains and NTA-utilizing *Chelatobacter heintzii* strains, the reader is referred to Table BXII.α.116 in the chapter on the genus *Chelatobacter*. Additional properties of *Chelatococcus asaccharovorans* are as follows. Substrates utilized for growth include acetate, alanine, aspartate, *n*-butanol, citrate, ethanol, S,S-ethylenediaminedisuccinate, glutamate, glycerol, glycine, lactate, malate, phenylalanine, propionate, proline, *n*-propanol, isopro-

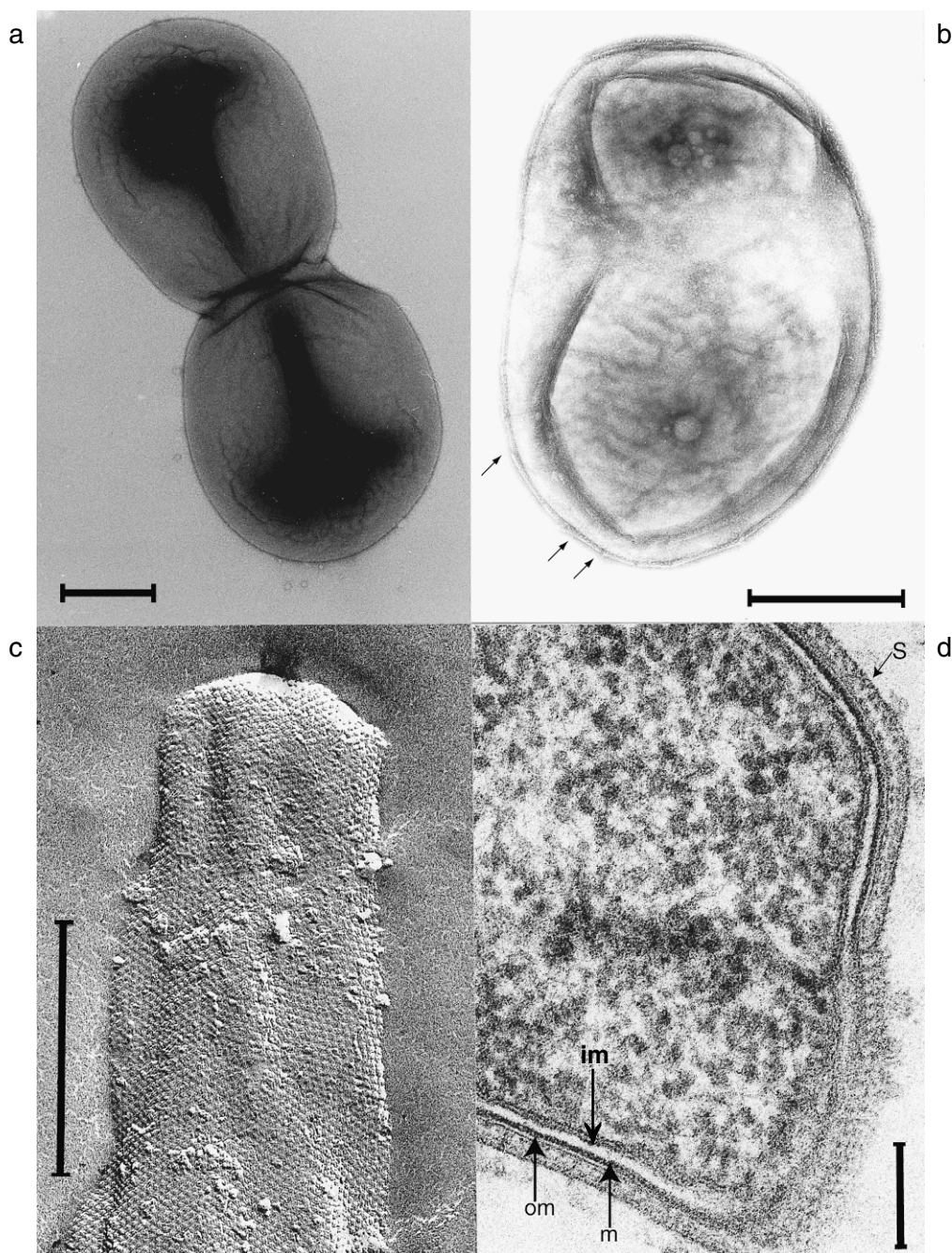


FIGURE BXII.α.169. Morphology of *Chelatococcus asaccharovorans* strains. (a), negatively stained cell of isolate TE 1 from the exponential growth phase, bar = 0.5 μ m; (b), negatively stained cell of isolate TE 2 demonstrating the proteinaceous surface layer; the cracks in the S-layer suggest that it is brittle in nature (arrows), bar = 0.5 μ m; (c), surface layer of strain TE 2 as revealed by deep-etching, note the paracrystalline arrangement of subunits, with a center-to-center distance of 16 nm, and the patchy appearance of the surface layer, bar = 0.5 μ m; (d), higher magnification of thin-sectioned cell of isolate TE 1 demonstrating the fine structure of the cell wall with inner membrane (im), murein layer (m), outer membrane (om), and proteinaceous surface layer (s), bar = 0.1 μ m.

panol, pyruvate, succinate, and serine. Substrates not utilized for growth include acetamide, adonitol, aniline, arabinose, L-arabitol, arginine, benzoate, *n*-decane, butyrate, D-(+)-cellobiose, cellulose, erythritol, dimethylformamide, dimethylsulfoxide, esculin, formate, fucose, D-(-)-fructose, galactose, gentiobiose, D-(+)-glucose, glycollate, glycyl-glycine, H₂/CO₂, inositol, lactose,

lysine, mannitol, D-(+)-mannose, malate, D-(+)-maltose, methane, methanol, methylacetamide, methyl-diethylamine, *N*-(2-acetamido)iminodiacetate, *N*-acetylglucosamine, oxalate, phenol, D-(+)-raffinose, rhamnose, D-(+)-ribose, D-(-)-sorbitol, sucrose, tetraethylammoniumchloride, triethanolamine, tris(hydroxymethyl)aminomethane, urea, xylitol, xylene, and D-(+)-xylose.

Other properties of *Chelatococcus asaccharovorans* strains include the following: no production of indole, H₂S, or acetoin (VP negative); no fermentation of glucose; no hydrolysis of starch or DNA and only weak hydrolysis of protein (skim milk); no dinitrogen fixation; no denitrification with NTA, glucose, or acetate; no acid production from glucose or ethanol; not acid fast; no growth on tellurite agar plates; no growth on mannitol (*Rhizobium*) agar; no gas from glucose; weak growth on gluconate. Growth occurs in the presence of 1% NaCl but not with 10% NaCl.

Metabolism and metabolic pathways A key nutritional feature of members of *Chelatococcus* is their ability to grow with the chelating agent NTA as the only source of carbon, energy, and nitrogen. (For a short overview on the use and importance of aminopolycarboxylates as metal-chelating agents, readers are referred to the chapter on *Chelatobacter*.) The current knowledge is summarized in Fig. BXII.α.133 in the chapter on the genus *Chelatobacter*. The data strongly support an identical catabolic route in both genera, i.e., initially, transport of NTA into the cell, a step which is most probably energy-dependent, and subsequently two enzymatic steps, catalyzed by a soluble NTA-mono-oxygenase complex and a membrane-bound dehydrogenase. These enzymes are sufficient to degrade NTA to the central metabolites glycine and glyoxylate.

NTA monooxygenase The first intracellular step is catalyzed by a monooxygenase (NTA-Mo). NTA-Mo was originally isolated from *Chelatobacter heintzii* ATCC 29600 as a two-component system consisting of component A (cA) and a FMN-containing protein component B (cB), with both components being present as dimers (Uetz et al., 1992). Using antibodies raised against cA isolated from *Chelatobacter heintzii* ATCC 29600, the presence of cA was confirmed in *Chelatococcus asaccharovorans* strains, whereas no cross reaction was observed in cell-free extracts of *Chelatococcus* strains with antibodies raised against the flavin-containing cB (Uetz et al., 1992). Later, cA purified from *Chelatococcus asaccharovorans* TE 2 was found to be virtually identical to the protein present in *Chelatobacter heintzii* ATCC 29600 (Uetz, 1992), i.e., only 1 out of the first 17 amino acids of cA was different (in position 3 Asn instead of Asp was present in cA purified from *Chelatococcus*). A hybrid enzyme composed of cA purified from *Chelatococcus* and cB from *Chelatobacter heintzii* ATCC 29600 exhibits the typical NTA-Mo activity known from strain ATCC 29600. According to Witschel et al. (1997), cA of *Chelatococcus* acts as a monooxygenase supplied with FMNH₂ by an independent FMN oxidoreductase. Recent data suggest that any FMN oxidoreductase can supply reduced FMN to the NTA-Mo (Witschel et al., 1997; Xu et al., 1997). Although not yet investigated in detail, the substrate specificity of NTA-Mo from *Chelatococcus* is probably as narrow as that found for NTA-Mo isolated from *Chelatobacter*, with NTA being the only substrate presently known (Uetz, 1992).

Iminodiacetate dehydrogenase A membrane-bound iminodiacetate dehydrogenase (IDA-DH) catalyses the second enzymatic step during biodegradation of NTA in *Chelatococcus asaccharovorans* strain TE 2, as also occurs in members of *Chelatobacter* (Uetz and Egli, 1993).

Regulation of NTA-degrading enzymes The enzyme system for NTA utilization is inducible (Uetz, 1992) but no detailed information is presently available. Nevertheless, other carbon sources do not seem to repress the synthesis of NTA-utilizing

enzymes, as growth in batch culture with mixtures of NTA plus acetate results in the simultaneous utilization of the two substrates concomitant with an enhanced growth rate (Egli et al., 1988; see also Table BXII.α.115 in the chapter describing the genus *Chelatobacter*).

Genetics Hybridization experiments with different gene probes from *Chelatobacter heintzii* ATCC 29600 strongly indicate that both the genes encoding *ntaA* and the ORF1—which probably encodes a regulatory protein—are present in *Chelatococcus asaccharovorans* strain TE 2 in a similar arrangement as that reported for the *Chelatobacter* strain (Knobel, 1997). This supports the data reported by Uetz (1992) on the N-terminal sequence of NTA-Mo purified from *Chelatococcus* strain TE 2. In contrast, only weak hybridization was found with probes directed towards *ntaB*, confirming the conclusion drawn by Uetz (1992), based on immunological evidence, that quite a different FMN oxidoreductase is supplying FMNH₂ to NTA-Mo in this strain.

Antigenic structure Polyclonal antibodies have been raised against whole cells (Wilberg et al., 1993) or isolated cell walls (Bally, 1994) of NTA-utilizing *Chelatobacter* and *Chelatococcus* strains. In Ouchterlony double diffusion or indirect immunofluorescence tests, the antisera raised against the two central NTA-utilizing *Chelatobacter* strains, i.e., *Chelatobacter heintzii* strains TE 6 and ATCC 29600, did not crossreact with cell homogenates from either *Chelatococcus asaccharovorans* strain. Similarly, the antiserum raised against *Chelatococcus asaccharovorans* strain TE 2 did not crossreact with homogenates of the two central strains of *Chelatobacter heintzii* (Wilberg et al., 1993). Crossreaction of *Chelatococcus* antibodies was also tested with a variety of bacteria that were expected to co-exist with *Chelatococcus* in the same habitat, and no crossreaction was found with any of the strains tested (for the strains tested, see the section on antigenic structure in the chapter on the genus *Chelatobacter*).

Antibiotics and drug resistance The susceptibility of *Chelatococcus asaccharovorans* strains TE 1 and TE 2 to a range of antibiotics was tested (Auling et al., 1993a; see Table BXII.α.117 in the chapter on the genus *Chelatobacter*). Both *Chelatococcus asaccharovorans* strains were resistant to cephaloridine, cephalixin, nalidixic acid, vancomycin, and trimethoprim-sulfamethoxazole, but sensitive to penicillin G, carbenicillin, most of the aminoglycosides tested (neomycin, kanamycin, gentamicin, tobramycin, except for streptomycin), and to tetracycline, doxycycline, erythromycin, rifampicin, polymyxin B, and novobiocin. The resistance of *Chelatococcus* strains to nalidixic acid, in combination with their resistance to vancomycin, might be used to isolate such strains with a medium containing NTA as the sole source of carbon and energy, as these two antibiotics would suppress growth of the faster-growing *Chelatobacter* strains.

Ecology Data reported so far indicate that cells of *Chelatococcus* are ubiquitously distributed in the aquatic environment, and that their number increases with both increasing eutrophication and temperature. Cells crossreacting with antibodies raised against *Chelatococcus asaccharovorans* TE 1 (α -Cc) have been detected in surface waters and wastewater (Wilberg et al., 1993; Bally, 1994). In surface waters, their numbers were in the order of 1×10^3 to 5×10^5 cells/l (0.03–3‰ of the total bacterial population). Cells of *Chelatococcus* have even been detected in mountain streams with no detectable NTA present and little chance of having been ever exposed to NTA; only in a cold pristine alpine stream were cells crossreacting with α -Cc not ob-

served (Bally, 1994). Generally, the number of crossreacting cells increased by several orders of magnitude in nutrient-rich environments such as polluted rivers or wastewater treatment plants. For example, in aerated reactors of two Swiss wastewater treatment plants (with NTA concentrations between 10 and 100 µg/l) numbers of *α-Ce*-crossreacting cells up to 2×10^{11} per liter were observed (in the range of one to a few percent of the total bacterial counts); many of the cells exhibited the typical diplococcoid shape (Bally, 1994). In this environment, members of *Chelatococcus* seem as numerous as strains of *Chelatobacter* or even more so (although these numbers have to be treated with caution considering possible crossreactivity of the antibodies used).

Polyamines and ubiquinones The two *Chelatococcus asaccharovorans* strains studied contained *sym*-homospermidine as the main polyamine compound, putrescine and spermidine as major components, and spermine as a minor component (Auling et al., 1993a). Ubiquinone Q-10 was present in both strains of *Chelatococcus asaccharovorans*.

Soluble protein patterns The soluble protein patterns of *Chelatococcus asaccharovorans* strains have been compared to those of *Chelatobacter heintzii* strains (Auling et al., 1993a). *Chelatobacter* and *Chelatococcus* strains exhibited clearly different patterns, whereas the soluble protein pattern of the two *Chelatococcus* strains tested were virtually identical.

ENRICHMENT AND ISOLATION PROCEDURES

Only two strains of *Chelatococcus* have been isolated to date. Both were isolated with a batch enrichment procedure using NTA as the only source of carbon, energy, and nitrogen (Egli, 1988). However, they were not as easily enriched as members of the genus *Chelatobacter*. The fact that the optimal growth temperature of *Chelatococcus* strains (35–37°C) is considerably higher than that of *Chelatobacter* strains, together with their resistance to vancomycin, might be a way to preferentially enrich members of this genus when using NTA as the only source of carbon and nitrogen for growth.

MAINTENANCE PROCEDURES

Strains can be maintained indefinitely in a freeze-dried condition. Alternatively, cultures grown in SM with NTA and amended with either glycerol (15%, v/v) or DMSO (50%, v/v) can be stored in liquid nitrogen. With either method, revival of cultures may take a while. Best results have been obtained by streaking cultures from liquid nitrogen directly onto SM agar plates containing NTA as the only carbon and nitrogen source. When maintaining cultures on selective NTA agar plates, it is recommended to transfer them to new plates at least every week, because growth leads to an increased pH in and around the colonies. Cells stored under such conditions exhibit long lag phases (up to several weeks) before they restart growing. Cultures grown in liquid media behave similarly. To avoid long lag times and erratic growth responses, it is best to transfer the cells to fresh medium when they are still growing exponentially. Excessive concentrations of NTA, combined with the low buffering capacity of the medium or lack of pH control, might lead to an early cessation of growth.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Growth with NTA as the only source of carbon, energy, and nitrogen is a key property of *Chelatococcus*. Consumption of NTA can be measured either by the disappearance of dissolved organic carbon (DOC) paralleled by the excretion of ammonia, or via the consumption of NTA. A HPLC method specially developed for the analysis of NTA in culture media and cell extracts has been described by Schneider et al. (1988).

DIFFERENTIATION OF THE GENUS *CHELATOCOCCUS* FROM OTHER GENERA

Differentiation of *Chelatococcus* from the closely related genospecies *Methylobacterium organophilum* and *Bradyrhizobium japonicum* (see taxonomic comments) can be easily done, based on the ability to grow with NTA as the sole carbon, nitrogen, and energy source, and the coccobacillary shape of the cells, their nonmotility, and the inability to grow on sugars (Green, 1992; Auling et al., 1993a, or see *Methylobacterium* in this *Manual*).

TAXONOMIC COMMENTS

The presence of ubiquinone Q-10 (Egli et al., 1988) and the characteristic polyamine pattern, with *sym*-homospermidine as the main polyamine and putrescine plus spermidine as major polyamines (Auling et al., 1993a), clearly allocate the members of the genus *Chelatococcus* to the *Alphaproteobacteria*. Sequencing of a 150-nucleotide 16S rRNA gene fragment (position 1220–1377) revealed nucleotides characteristic of the *Alphaproteobacteria* at specific positions, but a difference of 13 nucleotides between *Chelatococcus* and *Chelatobacter* indicated only a low degree of relationship between these genera (Auling et al., 1993a). This conclusion is supported by the absence of serological crossreaction between both genera of NTA-utilizing bacteria (Wilberg et al., 1993). Recently, analysis of complete 16S rRNA gene sequences revealed that *Methylobacterium organophilum* and *Bradyrhizobium japonicum* are more closely related to *Chelatococcus asaccharovorans* than to *Rhodopseudomonas acidophila* (C. Strömpl, personal communication). The next relative in this group for which 16S rRNA gene sequence data are available is *Beijerinckia indica*, with 94.5% sequence homology (C. Strömpl, personal communication).

A comparison of the complete 16S rDNA sequence of the type strain *Chelatococcus asaccharovorans* strain TE 2 with a partial sequence of strain TE 1 has so far shown identity of the two 16S rRNA genes (C. Strömpl, personal communication).

ACKNOWLEDGMENTS

We are indebted to Ernst Wehrli for supplying us with excellent electron micrographs. Also we thank C. Strömpl for supplying us with unpublished information on the phylogenetic position of *Chelatococcus asaccharovorans*. T.E. would like to thank H.U. Weilenmann and the students that have contributed to the study of NTA-utilizing microorganisms. Furthermore, the generous financial support of research on NTA in the laboratory of T.E. by grants from the Swiss National Science Foundation, Lever AG Switzerland and Unilever Port Sunlight, the Research Commission of ETH Zürich, and by EAWAG is gratefully acknowledged.

List of species of the genus *Chelatococcus*

1. ***Chelatococcus asaccharovorans*** Auling, Busse, Egli, El-Banna and Stackebrandt 1993b, 624^{VP} (Effective publication: Auling, Busse, Egli, El-Banna and Stackebrandt 1993a, 110.)

a.sac.cha.ro.vo'rans. Gr. pref. *a* not; Gr. n. *sacchar* sugar; L. v. *voro* to devour; M.L. part. adj. *asaccharovorans* not devouring sugars.

The characteristics are as described for the genus. To date, only two strains (TE1, DSMZ 6461; TE2, DSMZ 6462) represent the species.

The mol% G + C of the DNA is: 63 (T_m).

Type strain: TE 2, DSM 6462.

GenBank accession number (16S rRNA): AJ294349.

Genus III. *Methylocella* Dedysh, Liesack, Khmelenina, Suzina, Trotsenko, Semrau, Bares, Panikov and Tiedje 2000, 967^{VP}

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Me.thyl.o.cel'la. M.L. n. *methyl* the methyl group; L. n. *cella* a cell; M.L. n. *Methylocella* methyl-using cell.

Encapsulated, nonmotile straight or curved Gram-negative rods (0.6–1.0 × 1.0–2.5 µm). One large **poly-β-hydroxybutyrate granule** at each pole. **Form exospores**. Contain **intracytoplasmic membranes**. **Grow only on C₁ compounds using serine pathway**. Complete TCA cycle. **Fixes N₂**. Major fatty acids are C_{18:1} forms.

The mol% G + C of the DNA is: 61.2.

Type species: *Methylocella palustris* Dedysh, Liesack, Khmelenina, Suzina, Trotsenko, Semrau, Bares, Panikov and Tiedje 2000, 967.

FURTHER DESCRIPTIVE INFORMATION

A single species, *Methylocella palustris*, has been described; three independent isolates have been examined in detail. *Methylocella palustris* inhabits acidic peat bogs in northern Russia and Siberia (Dedysh et al., 1998a, b). Both the temperature range (10–20°C) and the pH range (4.5–7.0) for growth, as well as a requirement for dilute environments, reflect adaptation to this habitat (Dedysh et al., 2000).

Methylocella palustris is able to grow on methane and methanol as sole source of energy and carbon; the serine pathway is the route of carbon fixation. The organisms are able to fix N₂ in microaerobic environments and are able to use nitrate, ammonium ion, and organic nitrogen (Dedysh et al., 2000).

Intracytoplasmic membranes consist of spherical vesicles formed from the cytoplasmic membrane and located next to it. These membranes do not resemble either the vesicular discs found in type I methanotrophs or the laminar membranes found in type II methanotrophs (Dedysh et al., 2000).

Analysis of 16S rDNA sequences showed that *Methylocella palustris* is most closely related to *Beijerinckia indica* and *Rhodospseudomonas acidophila*; it is related to but clearly different from other type II methylobacteria (*Methylocystis* and *Methylosinus* spp.) in the *Alphaproteobacteria* (Dedysh et al., 1998a, 2000). Attempts

to PCR-amplify portions of the *pmoA* gene from *Methylocella palustris* isolates using two sets of primers that yielded specific products from *Methylococcus capsulatus* (type X methylobacter), *Methylosinus trichosporium* (type II methylobacter), and *Methylomicrobium album* (type I methylobacter) were unsuccessful. Southern hybridization of *Methylocella palustris* genomic DNA digests with a probe specific for the *Methylococcus capsulatus pmoA* gene produced only weak hybridization at low stringency, further indicating substantial genetic distance between *Methylocella palustris* and other methylobacteria in the *Alphaproteobacteria* (Dedysh et al., 1998a, 2000). An analysis of the fatty acid profiles of methylobacteria of all known metabolic types of methylobacteria (I, II, and X) also showed that *Methylocella palustris* was distinct from all other strains examined (Fang et al., 2000).

ENRICHMENT AND ISOLATION PROCEDURES

Enrichment and isolation procedures have been described by Dedysh et al. (1998a, b). Enrichment required multiple passages and was achieved in dilute liquid mineral medium under a 50% methane:air atmosphere with or without KNO₃ as a nitrogen source.

MAINTENANCE PROCEDURES

Strains can be maintained by subculturing monthly (Dedysh et al., 1998a, b).

DIFFERENTIATION OF THE GENUS *METHYLOCELLA* FROM OTHER GENERA

Dedysh et al. (2001) have developed FISH (fluorescence *in situ* hybridization) probes based on 16S rDNA sequences that can be used to distinguish *Methylocella palustris* from other methylobacteria in enrichment cultures and environmental samples.

List of species of the genus *Methylocella*

1. ***Methylocella palustris*** Dedysh, Liesack, Khmelenina, Suzina, Trotsenko, Semrau, Bares, Panikov and Tiedje 2000, 967^{VP}
pa.lus'tris. L. n. *palus* a bog; M.L. adj. *palustris* bog-inhabiting.

Description as for the genus with the following additional characteristics. Utilize methane and methanol. Growth optima at pH 5.5 and 20°C. Inhibited by 0.5% NaCl.

The mol% G + C of the DNA is: 61.2 (T_m).

Type strain: K, ATCC 700799.

GenBank accession number (16S rRNA): Y17144.

Family VII. **Bradyrhizobiaceae** *fam. nov.*

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Bra.dy.rhi.zo.bi.a'ce.ae. M.L. neut. n. *Bradyrhizobium* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Bradyrhizobiaceae* the *Bradyrhizobium* family.

The family *Bradyrhizobiaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Bradyrhizobium* (type genus), *Afipia*, *Agromonas*, *Blastobacter*, *Bosea*, *Nitrobacter*, *Oligotropha*, *Rhodoblastus*, and *Rhodopseudomonas*.

The family is phenotypically, metabolically, and ecologically diverse. It includes organisms that fix N₂, photosynthetic organisms, organisms capable of aerobic and/or anaerobic respiration, and human pathogens.

Type genus: Bradyrhizobium Jordan 1982, 137.

Genus I. Bradyrhizobium Jordan 1982, 137^{VP}

L. DAVID KUYKENDALL

Bra.dy.rhi.zo'bi.um. Gr. adj. *bradus* slow; M.L. neut. n. *Rhizobium* a bacterial generic name; M.L. neut. n. *Bradyrhizobium* the slow (growing) rhizobium.

Rods 0.5–0.9 × 1.2–3.0 µm. **Commonly pleomorphic under adverse growth conditions.** Usually contain granules of poly-β-hydroxybutyrate that are refractile by phase-contrast microscopy. **Nonsporeforming. Gram negative. Motile by one polar or subpolar flagellum.** Fimbriae have not been described. **Aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. Optimal temperature 25–30°C.** Optimal pH, 6–7, although lower optima may be exhibited by strains from acid soils. Colonies are circular, opaque, rarely translucent, white, and convex, and tend to be granular in texture; they do not exceed 1.0 mm in diameter in less than 5–6 days incubation on ALEG medium. **Turbidity develops only after 3–4 days in agitated broth. Generation times are 9–18 h.** Chemoorganotrophic, utilizing a range of carbohydrates and salts of organic acids as carbon sources, without gas formation; arabinose and other pentoses are preferred carbon sources. Cellulose and starch are not utilized. Produce an alkaline reaction in mineral salts medium containing mannitol and/or many other carbohydrates. **Growth on carbohydrate media is usually accompanied by extracellular polysaccharide slime production particularly with glycerol, gluconate, or mannitol.** Some strains can grow chemolithotrophically in the presence of H₂, CO₂, and low levels of O₂. Ammonium salts, usually nitrates, and some amino acids, can serve as nitrogen sources. Peptone is poorly utilized (except for strains isolated from *Lotononis*). Casein and agar are not hydrolyzed. There is usually no requirement for vitamins with the rare exception of biotin, which also may be inhibitory to some strains. 3-Ketoglycosides are not produced (Bernaerts and De Ley, 1963). **The organisms are characteristically able to enter the root hairs of tropical-zone and some temperate-zone leguminous plants (family Leguminosae) and incite the production of root nodules, in which the bacteria occur as intracellular nitrogen-fixing symbionts.** Some strains, especially *B. elkanii*, fix nitrogen in the free-living state when examined under special conditions.

The mol% G + C of the DNA is: 61–65.

Type species: Bradyrhizobium japonicum (Kirchner 1986) Jordan 1982, 137 (“*Rhizobacterium japonicum*” Kirchner 1986, 221; *Rhizobium japonicum* (Kirchner 1986) Buchanan 1926, 90.)

FURTHER DESCRIPTIVE INFORMATION

In young cultures, cells are short rods but in older cultures or under adverse growth conditions, such as low concentrations of calcium or magnesium, cells are commonly pleomorphic (swollen and elongated). Older cells stain to give a banded appearance

because of large accumulations of poly-β-hydroxybutyrate. Within the root nodules the bacteroids are rod shaped and slightly swollen but not branched or highly distorted, and they contain polyphosphate inclusions as well as poly-β-hydroxybutyrate accumulations.

All strains produce large amounts of water-soluble extracellular polysaccharide. The main constituent is acidic heteropolysaccharide (80–90%); the remainder is neutral β-1,2-, β-1,3-, and α-1,2-glucans (Breedveld and Miller, 1994). About six different types of acidic heteropolysaccharides have been noted in an examination of 30 or more strains of *Bradyrhizobium* and possess three distinctive features: (a) heterogeneity in composition and structure, (b) the frequent presence of methylated sugars (Dudman, 1976, 1978; Kennedy and Bailey, 1976), and (c) the presence of D-galacturonic acid (Dudman, 1976) in some strains.

Young cells are motile with one polar or subpolar flagellum. Growth on carbohydrate-containing solid media is clear, translucent, or opaque white with a tendency to become tan on prolonged incubation. Colonies of bacteria from *Lotononis bainesii* are pink to deep red but those from *Lotononis angolensis* are colorless.

Most strains grow on a chemically defined minimal medium with arabinose as a carbon source and NH₄Cl as the sole source of nitrogen (Kuykendall, 1987). All strains grow on a buffered mineral salts medium containing yeast extract and either glucose, galactose, gluconate, glycerol, fructose, or arabinose. Disaccharides such as lactose and sucrose are usually not used. Colony-type derivatives differing in symbiotic nitrogen fixation ability and carbon source utilization occur (Kuykendall and Elkan, 1976) (Fig. BXII.α.170).

As with *Rhizobium*, *Bradyrhizobium* strains lack the ability to absorb Congo red from a yeast extract–mannitol–mineral salts medium containing this dye (0.0025% final concentration). The colonies are colorless white or very faintly pink colonies, whereas most other soil bacteria produce colonies that are red on this differential medium.

The optimal temperature for most strains is 25–30°C. The maximum growth temperature ranges from 33–35°C, with many strains failing to grow above 34°C. Usually acid-tolerant with most strains growing at pH 4.5. Over 30% of the strains will grow at pH 4.0 and a few as low as pH 3.5. Growth usually does not occur above pH 9.0.

An alkaline reaction is produced in litmus milk without the production of a clear, upper serum zone.

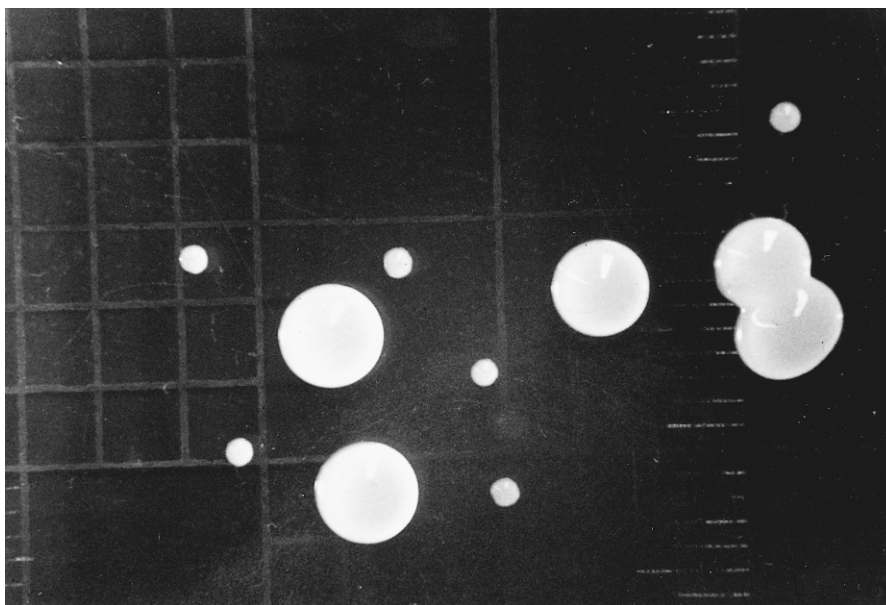


FIGURE BXII.α.170. *Bradyrhizobium japonicum* strain USDA 110 derivatives I-110 (smaller colonies) and L1-110 (larger colonies) after 14 d incubation at 30°C grown on HM salts agar (Cole and Elkan, 1973) with yeast extract (0.25 g/l), L-arabinose (0.5 g/l), and D-mannitol (5.0 g/l).

Bradyrhizobium strains fail to grow in media containing 2% NaCl, do not produce H_2S , and do not form a precipitate in calcium glycerophosphate medium (Hofer, 1941). Penicillinase production is common.

The Entner–Doudoroff pathway is employed in carbohydrate degradation (Martínez-De Drets and Arias, 1972; Mulongoy and Elkan, 1977) with the simultaneous operation of the Embden–Meyerhof–Parnas pathway (Mulongoy and Elkan, 1977). Low levels of fructose-1,6-bisphosphate aldolase are present, however. $NADP^+$ -dependent 6-phosphogluconate dehydrogenase is not present but an NAD-linked 6-phosphogluconate dehydrogenase occurs, suggesting operation of a new pathway. The tricarboxylic acid cycle operates in both free-living cells (Keele et al., 1969; Mulongoy and Elkan, 1977) and in symbiotic bacteroids from root nodules (Stovall and Cole, 1978). A pathway for the direct oxidation of gluconate by the tricarboxylic acid cycle, via 2-keto- and 2,5-diketogluconate and α -ketoglutarate, has been described by Keele et al. (1970). Many differences in carbon metabolism between fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* were described by Elkan and Kuykendall (1982); they seem especially significant since these genera are now in separate families.

The ability to take up H_2 was shown by the laboratory of Dr. Harold J. Evans to be a key determining factor in the efficiency of symbiotic nitrogen fixation, because without this capability the nodules evolve large quantities of H_2 as a byproduct of the nitrogenase enzyme action. Some strains do not evolve any H_2 ; such strains possess an active uptake hydrogenase, which also enables them to grow chemolithotrophically in an atmosphere of $H_2/CO_2/O_2$ (10:15:1) (the balance being N_2). Under such conditions, ribulose biphosphate carboxylase is primarily responsible for the fixation of CO_2 (Hanus et al., 1979; Lepo et al., 1980). Mutants unable to grow chemolithotrophically fall into several classes, including those impaired in H_2 uptake and those deficient in CO_2 uptake (Maier, 1981).

Nitrogenase activity by free living cells occurs in certain strains but only in media containing selected carbon sources and under a low level of oxygen (Keister, 1975; Kurtz and LaRue, 1975; McComb et al., 1975; Pagan et al., 1975).

Auxotrophic mutants (Kuykendall, 1981) were characterized by Wells and Kuykendall (1983) and Kummer and Kuykendall (1989). Most tryptophan-requiring mutants do not nodulate soybeans but those that specifically lack only tryptophan synthetase do form nodules. An indole glycerol phosphate-requiring mutant, strain TA-11, was discovered to produce phenotypic revertants having the capacity to quantitatively form more nodules (Hunter and Kuykendall, 1990; Kuykendall and Hunter, 1991).

Host specificity, nitrogen fixation (*nif*) and nodulation (*nod*) genes have been analyzed (Kaluza and Hennecke, 1984; Lamb and Hennecke, 1986; Nieuwkoop et al., 1987; Banfalvi et al., 1988; Gottfert et al., 1990a, b, 1992; Sadowsky et al., 1991; Wang and Stacey, 1991; Barbour et al., 1992; Dockendorff et al., 1994; and Stacey et al., 1994). Unlike the fast-growing *Rhizobiaceae*, *Bradyrhizobium* strains have only one rRNA operon (Kundig et al., 1995). A correlated physical and genetic map is available and all *nif* and *nod* genes are contained in a large but discrete region of the 8700 kb chromosome (Kundig et al., 1993). Kaneko et al. (2002) recently reported the complete polynucleotide sequence of *B. japonicum* USDA 110. Much is known about the function, organization and sequence of symbiosis-controlling genes (see reviews by Schultze et al., 1994; Stacey et al., 1995; van Rhijn and Vanderleyden, 1995). Kaluza et al. (1985) demonstrated the presence of a repeated DNA sequence, RS α , clustered around the symbiosis-controlling region of the genome, and Minamisawa et al. (1998) recently described some new strains with an unusually high RS α copy number.

Bacteriophages active against *Bradyrhizobium* have been isolated from soil (Hashem et al., 1986). Bacteriocins have been reported (Roslycky, 1967).

Bradyrhizobium strains are often resistant to a number of anti-

biotics (tetracyclines, streptomycin, penicillin G, viomycin, vancomycin), but are said to be more sensitive to growth inhibitors such as D-alanine or ethidium bromide (Jordan, 1984c).

Bradyrhizobium strains cause nodule production on *Glycine* (soybean), *Vigna* (cowpea), *Macroptilium* (siratiro), certain species of *Lotus*, and a wide variety of leguminous plants that are also nodulated by *Mesorhizobium loti*, such as *Acacia*. In fact there appear to be three distinct groups of *Acacia* species: one group nodulated by *M. loti*, one group nodulated by *Bradyrhizobium* sp. (*Acacia*) and one group nodulated by both of these species (Dreyfus and Dommergues, 1981). As with *Rhizobium*, *Bradyrhizobium* strains produce specific Nod Factors thought to control host specificity (Dénarié et al., 1992; Carlson et al., 1993; Stacey et al., 1995).

A highly specific nodulation of the nonleguminous plant *Parasponia* (*Trema*) is caused by a strain of *Bradyrhizobium* (Trinick, 1973, 1976). This is the only completely validated instance of plant nodulation that occurs outside of the Leguminosae.

ENRICHMENT AND ISOLATION PROCEDURES

Isolation from root nodules and soil is by the techniques described for the genus *Rhizobium* of the *Rhizobiaceae*.

MAINTENANCE PROCEDURES

A suitable maintenance medium is A1EG (Kuykendall, 1987) or arabinose enriched (0.1% yeast extract) gluconate, as given by Kuykendall et al. (1988). Storage recommendations are the same as given for *Rhizobium*, except *Bradyrhizobium* persists on agar slants in tightly closed screw-capped tubes for more than five years at 4°C.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

For antibiotic resistance determinations, Petri dishes containing about 25 ml of A1EG agar medium containing a specific concentration of antibiotic are streaked for isolation with about 10 µl of a fresh 3–5 day-old broth culture. Growth of isolated colonies is determined visually after 10 d incubation. Resistance to a particular concentration is defined as the ability of a strain to form colonies at that concentration.

DIFFERENTIATION OF THE GENUS *BRADYRHIZOBIUM* FROM OTHER GENERA

The ability to fix nitrogen symbiotically in association with legumes is the primary characteristic that distinguishes *Bradyrhizobium* from other closely related genera in the new family *Bradyrhizobiaceae*. It is easily differentiated from the not so closely-related, relatively fast-growing, legume-nodulating microsymbionts in *Mesorhizobium*, *Rhizobium*, and *Azorhizobium* (Table BXII.α.143).

TAXONOMIC COMMENTS

In the first edition of *Bergey's Manual of Systematic Bacteriology*, *Bradyrhizobium* was still in the family *Rhizobiaceae*. The genus *Rhizobium* had previously been subdivided into two groups, distin-

guished by their growth rate in yeast extract–mannitol–mineral salts medium, flagellar arrangement, DNA base composition and the genera of host plants nodulated (Jordan and Allen, 1974). Jordan (1982) transferred the slow-growing root nodule bacteria into a new genus, *Bradyrhizobium*, separate from the fast growing, acid-producing, nodulating bacteria, which subsequently were placed in genera of their own. This revision resulted from numerous studies involving numerical taxonomy (Graham, 1964; Moffett and Colwell, 1968), DNA base ratios (Wagenbreth, 1961; De Ley and Rassel, 1965), nucleic acid hybridization (Heberlein et al., 1967; Gibbins and Gregory, 1972), cistron similarities (De Smedt and De Ley, 1977), serology (Graham, 1963; Vincent and Humphrey, 1970; Humphrey et al., 1973; Vincent, 1977), composition of extracellular gum (Dudman, 1976; Kennedy, 1976; Kennedy and Bailey, 1976; Dudman, 1978), carbohydrate metabolism (Martínez-De Drets and Arias, 1972), bacteriophage sensitivity (Napoli et al., 1980), antibiotic sensitivity (Strzelcowa, 1968), protein composition (Roberts et al., 1980), and type of bacteroid inclusion bodies (Craig et al., 1973). Based on 16S rRNA sequences analyses, *Bradyrhizobium* is now grouped with phylogenetically related organisms in a new separate family, the *Bradyrhizobiaceae*.

Three species of *Bradyrhizobium* are presently recognized; at least 16 genomospecies have also been isolated (Lafay and Burdon, 1998).

In the first edition of *Bergey's Manual of Systematic Bacteriology*, the genus *Bradyrhizobium* had only one recognized species, *B. japonicum*. However, DNA–DNA hybridization studies by Hollis et al. (1981) had earlier suggested that a subset of strains in *B. japonicum* could perhaps be separated into a distinct species, and that two DNA homology groups of bona fide *B. japonicum* existed as well. *B. elkanii* (Kuykendall et al., 1992, 1993b; Devine and Kuykendall, 1996) was named to recognize a species of *Bradyrhizobium* bacteria clearly distinct from *B. japonicum*. Extra-slow-growing strains from alkaline soils were then named *Bradyrhizobium liaoningense* Xu et al. (1995).

The taxonomic position of nodule bacteria from the pasture legume *Lotononis* is uncertain (Norris, 1958). Isolates from *L. bainesii* are red because of an intracellular red carotenoid pigment, although isolates from *L. angolensis* are nonpigmented. As with other strains of *Bradyrhizobium*, these strains are monotrichous, grow slowly, and produce an alkaline reaction and no serum zone in litmus milk; however, cultured cells appear as enlarged, banded ovoids. Resistance of these strains to ultraviolet light is greater than that exhibited by other strains of *Bradyrhizobium* or of *Rhizobium* (Law, 1979). They also utilize peptone, are not recognized by antisera that detect other slow growers or species of *Rhizobium*, and the mol% G + C of the DNA is 68–69 (*T_m*) (Godfrey, 1972). Nodulation by these strains is extremely specialized. Nodulation by *Lotononis* spp. and *Macroptilium atropurpureum* is effective, whereas by selected species of *Aeschynomene* and *Crotalaria* is ineffective.

Comments concerning *Sinorhizobium fredii* (Scholla and Elkan, 1984; Chen et al., 1988b) are presented under Taxonomic Com-

TABLE BXII.α.143. Phenotypic differentiation of *Bradyrhizobium* from other genera of nodule-forming, nitrogen-fixing legume microsymbionts

Characteristic	<i>Bradyrhizobium</i>	<i>Azorhizobium</i>	<i>Mesorhizobium</i>
Grows <i>in vitro</i> on fixed N	—	+	—
Fixes nitrogen <i>ex planta</i>	+	+	—
Slow doubling times, >8h	+	—	—

ments for the genus *Rhizobium*. These fast growing root-nodule bacteria are capable of producing effective nodules on *Glycine soja* and *G. max* cv Peking, but ineffective nodules on commercial lines of soybean.

ACKNOWLEDGMENTS

The author appreciates Dr. Bob Davis, Research Leader of the Molecular Plant Pathology Laboratory, for support and encouragement, thanks Dr.

Thomas E. Devine whose collaboration and friendship made a real difference, and was indebted to Professors Gerald Elkan and Hauke Hennecke who were mentors. Drs. Fawzy Hashem, Babita Saxena, Jim Hunter, Greg Upchurch, Mulongoy Kalemari, Gail Hollowell, Robin Kummer, Margaret Roy, Ketan Shah, Matthias Hahn, Bill Gillette, Susan Wells, and Tom Wacek all helped investigate the properties of *Bradyrhizobium*.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *BRADYRHIZOBIUM*

While *B. elkanii* and *B. japonicum* are readily distinguished by a variety of phenotypic analyses, there is a paucity of data available on qualitative differences between *Bradyrhizobium liaoningense* and

the two previously named species (Table BXII.α.144). This highlights the need for phenotypic characterization of the third species.

List of species of the genus *Bradyrhizobium*

1. ***Bradyrhizobium japonicum*** (Kirchner 1896) Jordan 1982, 137^{VP} ("*Rhizobacterium japonicum*" Kirchner 1896, 221; *Rhizobium japonicum* (Kirchner 1896) Buchanan 1926, 90.) *ja.po' ni.cum.* M.L. adj. *japonicum* pertaining to Japan.

The characteristics are as described for the genus and as indicated in Table BXII.α.144. Whole cell fatty acid content is 1.3% C_{16:1 ω7c}, 3.6% C_{16:1}, 8.8% C_{16:0}, 1.2% C_{19:0 cyclo}, and 81.2% C_{18:1}, when grown on AIEG agar medium at 30°C for 6 days. Resistant to trimethoprim at 50 mg/l and to vancomycin at 100 mg/l but sensitive to nalidixic acid (50 mg/l), tetracycline (100 mg/l), streptomycin (100 mg/l), erythromycin (250 mg/l), chloramphenicol (500 mg/l), rifampicin (500 mg/l), and carbenicillin (500 mg/l). Does not grow on Beringer's TY Medium that is used to grow *Rhizobium*. The exocellular polysaccharide contains glucose, mannose, galacturonic acid, and galactose. Denitrification positive. Bacteroids in root nodules are usually viable when plated onto media. Cells of *B. japonicum* have one polar or subpolar flagellum. The species normally causes the formation of root nodules on species of *Glycine* (soybean) (see Fig BXII.α.171) and on *Macroptilium atropurpureum* (siratro). Some strains express hydrogenase activity with the soybean host and thus are more efficient in symbiotic nitrogen fixation.

The mol% G + C of the DNA is: 61–65 (*T_m*).

Type strain: USDA 6, ATCC 10324, DSMZ 30131.

GenBank accession number (16S rRNA): U69638, X87272.

2. ***Bradyrhizobium elkanii*** Kuykendall, Saxena, Devine, and Udell 1993b, 398^{VP} (Effective publication: Kuykendall, Saxena, Devine, and Udell 1992, 504.)

el.ka' ni.i. M.L. n. *elkanii* of G.H. Elkan.

Most characteristics are as described for the genus and as indicated in Table BXII.α.144. Unlike *B. japonicum*, *B. elkanii* readily fixes nitrogen *ex planta*, strains generally produce indole acetic acid in liquid or broth cultures, they produce at least some growth on TY medium, and they generally grow at 34°C or higher temperatures. Strains are better established in the southeastern United States than the rest of the country. Whole cell fatty acid content is 0.5% C_{16:1}, 11.1% C_{16:0}, 0.8% C_{17:0 cyclo}, 24.7% C_{19:0 cyclo}, and 62.3% C_{18:1}, when grown on AIEG agar medium at 30°C for 6 days. Like *B. japonicum*, it is resistant to trimethoprim at 50 mg/l and to vancomycin at 100 mg/l. Unlike *B. japonicum*, *B. elkanii* is resistant to nalidixic acid (50 mg/l), tetracycline (100 mg/l), streptomycin (100 mg/l), erythromycin (250 mg/l), chloramphenicol (500 mg/l), rifampicin (500 mg/l), and carbenicillin (500 mg/l). The exocellular polysaccharide contains rhamnose and 4-*o*-methyl-glucuronic acid. Cells lack nitrite reductase activity but can possess the ability to reduce nitrate to nitrite. The species normally causes the formation of root nodules on species of *Glycine* (soybean), non-nodulating *rj1rj1* soybean, black-eyed peas (*Vigna*), mungbean, and on *Macroptilium atropurpureum* (siratro). Unlike *B. japonicum*, *B. elkanii* often produces rhizobitoxine-induced chlorosis on sensitive soybean cultivars (Devine et al., 1988; 1990). Strains often are hydrogenase positive on *Vigna* but not *Glycine*, suggesting more symbiotic affinity or compatibility with the former than the latter. *B. elkanii* may have originated on a continent other than Asia, where *B. japonicum* originates. Bottomley et al. (1994) used

TABLE BXII.α.144. The differential characteristics of the species of *Bradyrhizobium*^a

Characteristic	<i>Bradyrhizobium japonicum</i>	<i>Bradyrhizobium elkanii</i>	<i>Bradyrhizobium liaoningense</i>
Extra-slow growth (>18h)	—	—	+
Antibiotic resistance	—	+	—
Broad host range	—	+	NR
EPS has rhamnose	—	+	NR
<i>ex planta</i> Nitrogenase	L	H	NR
C _{19:0 cyclo}	H	L	NR
Nitrite reduction	+	—	NR
Indole-3-acetic acid produced in broth	—	+	NR
L-Rhamnose utilization	—	+	NR
D-Glucosamine utilization	—	+	NR
D-Alanine utilization	—	+	NR
Malonic acid utilization	+	—	NR

^aFor symbols, see standard definition; L, low; H, high; NR, not reported.

multilocus enzyme electrophoresis to confirm the genetically distinct lineages of *B. japonicum* and *B. elkanii*. The data of Barrera et al. (1997) indicate that many Mexican *Lupinus*-nodulating bacteria belong to *B. elkanii* but some are *B. japonicum*.

The mol% G + C of the DNA is: 61–65 (T_m).

Type strain: USDA 76, ATCC 49852, DSMZ 11554.

GenBank accession number (16S rRNA): U35000.

3. ***Bradyrhizobium liaoningense*** Xu, Ge, Cui, Li and Fan 1995, 706^{VP}

li.a.o.ning.en'se. M.L. adj. *liaoningense* referring to a province in China where they originated.

This species consists of extra-slow-growing bradyrhizobia from alkaline soils. Colonies are only 0.2–1.0 mm on YMA medium (recipe given in *Rhizobium* chapter) after 7–14 days. Unlike *B. elkanii*, this species is more susceptible to antibiotics at low concentrations. Numerical taxonomy and serotyping were also used to establish an identity for this species apart from the two previously described species. A representative strain, 2062, had less than 25% DNA homology with strains of either *B. japonicum* or *B. elkanii*.

The mol% G + C of the DNA is: 61–65 (T_m).

Type strain: 2281.

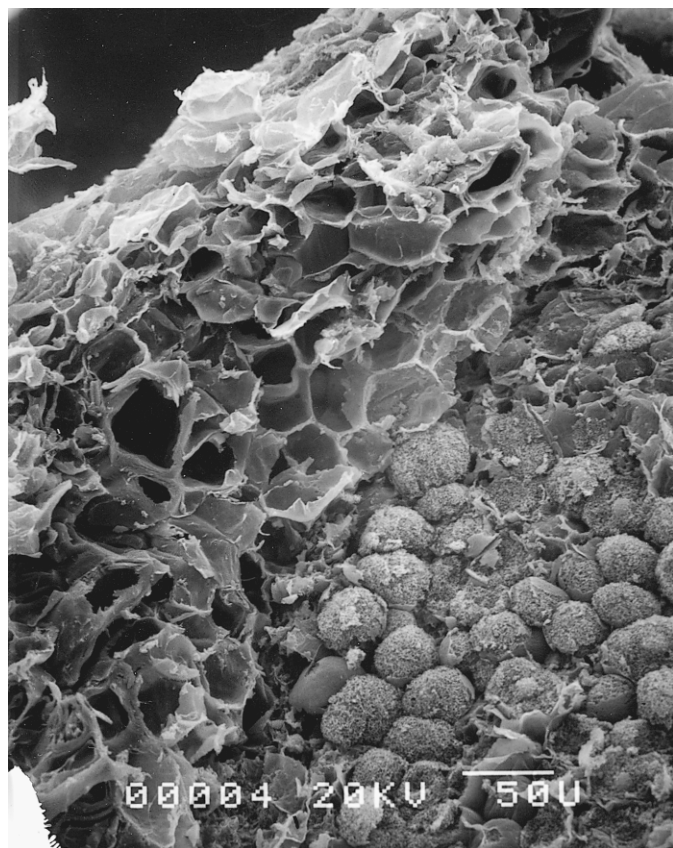
GenBank accession number (16S rRNA): X86065.

Additional Remarks: This sequence is not from the type strain.

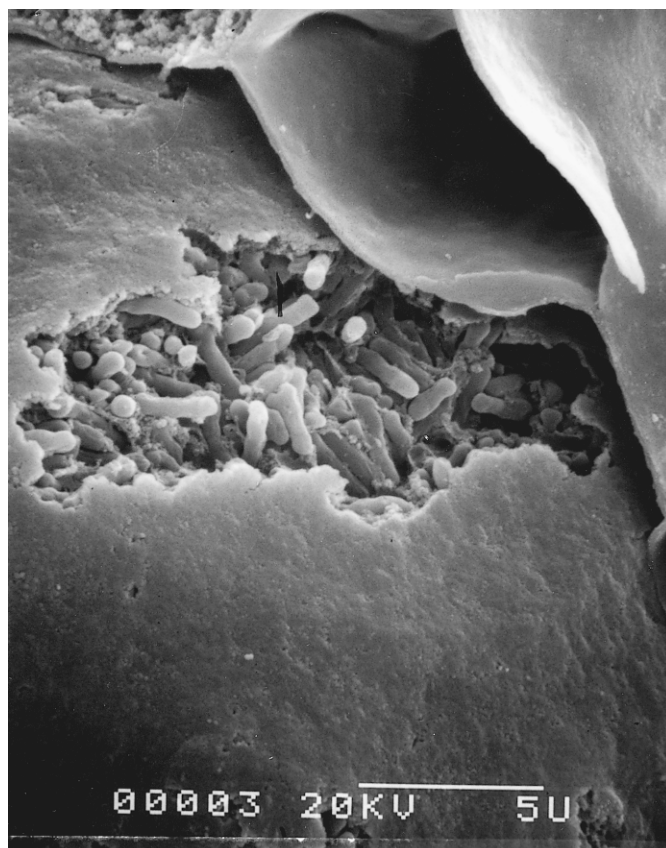
Other Organisms

Ladha and So (1994) used numerical taxonomy to show that *Bradyrhizobium* sp. (*Aeschynomene*) strains may constitute a separate taxon from *B. japonicum* and *B. elkanii* but FAME and 16S rRNA sequence data did not support this hypothesis (So et al., 1994). Wong et al. (1994) demonstrated that *Bradyrhizobium* spp. (*Aeschynomene*) are closely related to *B. japonicum*. Other *Bradyrhizobium* species are known to occur but have not yet been named. For example, the fatty acid data of Graham et al. (1995) clearly document distinct microorganisms. Dupuy et al. (1994) established that *Acacia*-nodulating *Bradyrhizobium* strains in Africa cor-

responded to either *B. japonicum*, *B. elkanii*, or one or more as yet unnamed *Bradyrhizobium* spp. Related organisms cause nodule production on certain species of *Lotus* (*L. uliginosus* and *L. pedunculatus*) and also on *Vigna* and species of *Lupinus*, *Ornithopus*, *Cicer*, *Sesbania*, *Leucaena*, *Mimosa*, *Lablab*, and *Acacia*, which are also nodulated by the relatively fast-growing species *Mesorhizobium loti*. Such strains also usually nodulate *Macroptilium* and, more rarely, *Glycine*. It is suggested that until such time as further species or biovars are created within the genus *Bradyrhizobium*, these organisms (other than the species described above) be desig-



A



B

FIGURE BXII.α.171. Scanning EM of the freeze-fractured interior of a root nodule formed by *Bradyrhizobium japonicum* strain I-110 on soybean. (A) Plant cells filled with viable bacteria are in the center portion. (B) The cell wall of a bacteria-filled cell was partially torn away, showing the rod-shaped bacteria within. (EM micrographs were taken in the laboratory of Dr. Bill Wergin at Beltsville, MD.)

nated as *Bradyrhizobium* sp. with the name of the appropriate host plant given in parenthesis immediately following; e.g., *Bradyrhizobium* sp. (*Acacia*). A number of *Bradyrhizobium* sp. (*Arachis*)

strains were found to be closely related to *B. japonicum* (Van Rossum et al., 1995).

Genus II. *Afipia* Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger 1992, 327^{VP} (Effective publication: Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger, 1991, 2457.)

ROBBIN S. WEYANT AND ANNE M. WHITNEY

A. fip' i.a. L. fem. n. *Afipia* derived from the abbreviation AFIP, for the Armed Forces Institute of Pathology, where the type strain of the type species was isolated.

Gram-negative rods in the *Alphaproteobacteria*. **Motile** by means of one or two polar, subpolar, or lateral flagella. **Optimal growth is obtained at 30°C** and growth does not occur at or above 42°C. Grows well on buffered charcoal yeast extract (BCYE) agar and in nutrient broth with 0% added NaCl. Growth does not occur in nutrient broth with 6% added NaCl and is not enhanced by increased CO₂. Colonies are gray-white, glistening, convex and opaque, 0.5–1.5 mm in diameter at 72 hours of incubation at 30°C on BCYE agar. Nonfermentative metabolism; acid is not produced from D-glucose, lactose, maltose, or sucrose. **Positive for oxidase, urease, and litmus milk alkalization.** Negative for hemolysis, gas production from nitrate, production of indole and H₂S (triple sugar iron agar method), and hydrolysis of gelatin and esculin. Isolated from potable water. Associated with human infection. Interrelatedness of species by DNA–DNA hybridization is 12–69%.

The mol% G + C of the DNA is: 61.5–69.

Type species: *Afipia felis* Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger 1992, 327 (Effective publication: Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger, 1991, 2458.)

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic treatment On the basis of DNA hybridization and phenotypic characterization, the genus *Afipia* currently contains 3 named species, *Afipia felis*, *A. broomeae*, and *A. clevelandensis* as well as three unnamed genomospecies (Brenner et al., 1991a). When compared with other members of the *Alphaproteobacteria* by 16S rDNA sequence analysis, *Afipia* species are intermingled with species of *Rhodopseudomonas*, *Bradyrhizobium*, *Nitrobacter*, and *Blastobacter* as shown in the phylogenetic tree in Fig. BXII.α.172. The 16S rRNA genes of *A. felis*, *A. broomeae*, and *A. clevelandensis* share 97.6–98.5% similarity to each other, and 97.0–98.5% to *Afipia* genomospecies 3, *Rhodopseudomonas palustris*, *Nitrobacter winogradskyi*, *Nitrobacter hamburgensis*, "*Bradyrhizobium lupini*", *Bradyrhizobium japonicum*, and *Blastobacter denitrificans*. The high degree of sequence similarity supports Willems and Collins' proposal that these species could be classified as members of a single genus (Willems and Collins, 1992). The 16S rRNA sequences for *Afipia*

genomospecies 1 and 2, however, are 95–95.9% similar to those of the named *Afipia* species and *Afipia* genomospecies 3.

Eleven additional genomospecies have been tentatively included in the genus *Afipia* based on phenotypic characteristics, chemotaxonomy, and DNA relatedness (Weyant et al., unpublished data). These strains are described in the section Other Organisms.

Cell and flagella morphology *Afipia* cells can be visualized using the Gram stain and other common staining techniques. Gram-stained preparations of 48-hr BCYE cultures show short to medium-length (>1.0 µm) Gram-negative rods of medium width (>0.5 µm). Cells are randomly distributed and do not form chains or clusters. Motility is achieved by one or two polar or subpolar flagella. Flagella may be easily visualized using the Ryu method (Ryu, 1937; Kodaka et al., 1982). Characteristic Gram and flagella stains of *Afipia* species have been illustrated by English et al. (1988) and Weyant et al. (1995).

Cellular fatty acid (CFA) composition and ubiquinone content All *Afipia* species share a unique CFA profile of predominately branched fatty acids (C_{16:1 ω7c}, C_{18:1 ω7c}, and C_{B₁-19:1}). The high amount of C_{B₁-19:1} (10–23%) is unique to *Afipia*. All *Afipia* species also contain C_{17:0 cyclo} and/or C_{19:0 cyclo ω7c} in proportions ranging from 1 to 15% (Moss et al., 1990a; Weyant et al., 1995). Ubiquinone 10 is the major isoprenoid ubiquinone of *Afipia* (Moss et al., 1991).

Colonial and cultural characteristics *Afipia* strains grow in various common growth media, including heart infusion agar with 5% rabbit blood, trypticase soy agar with 5% sheep blood, tryptone glucose yeast extract agar, and BCYE. Broth media that support *Afipia* growth include heart infusion broth and nutrient broth. Growth occurs at 25, 30, and 35°C, but not at or above 42°C. Optimal growth is at pH 6.8 (Müller, 1995). Growth occurs under aerobic conditions and is not enhanced by increased CO₂. Growth does not occur in nutrient broth containing 6% NaCl and only rarely occurs on MacConkey agar. Growth is weakly enhanced by ferric pyrophosphate, cysteine-HCl, and α-ketoglutarate (Müller, 1995).

Optimal growth is observed on BCYE agar under aerobic conditions at an incubation temperature of 30–32°C. Growth is relatively slow, with colonies appearing between 48 and 72 hours of incubation under optimal conditions. Colonies are gray-white, glistening, convex, and opaque, with smooth edges. Colony diameters range from 0.5 to 1.5 mm. No hemolysis is observed on blood agar.

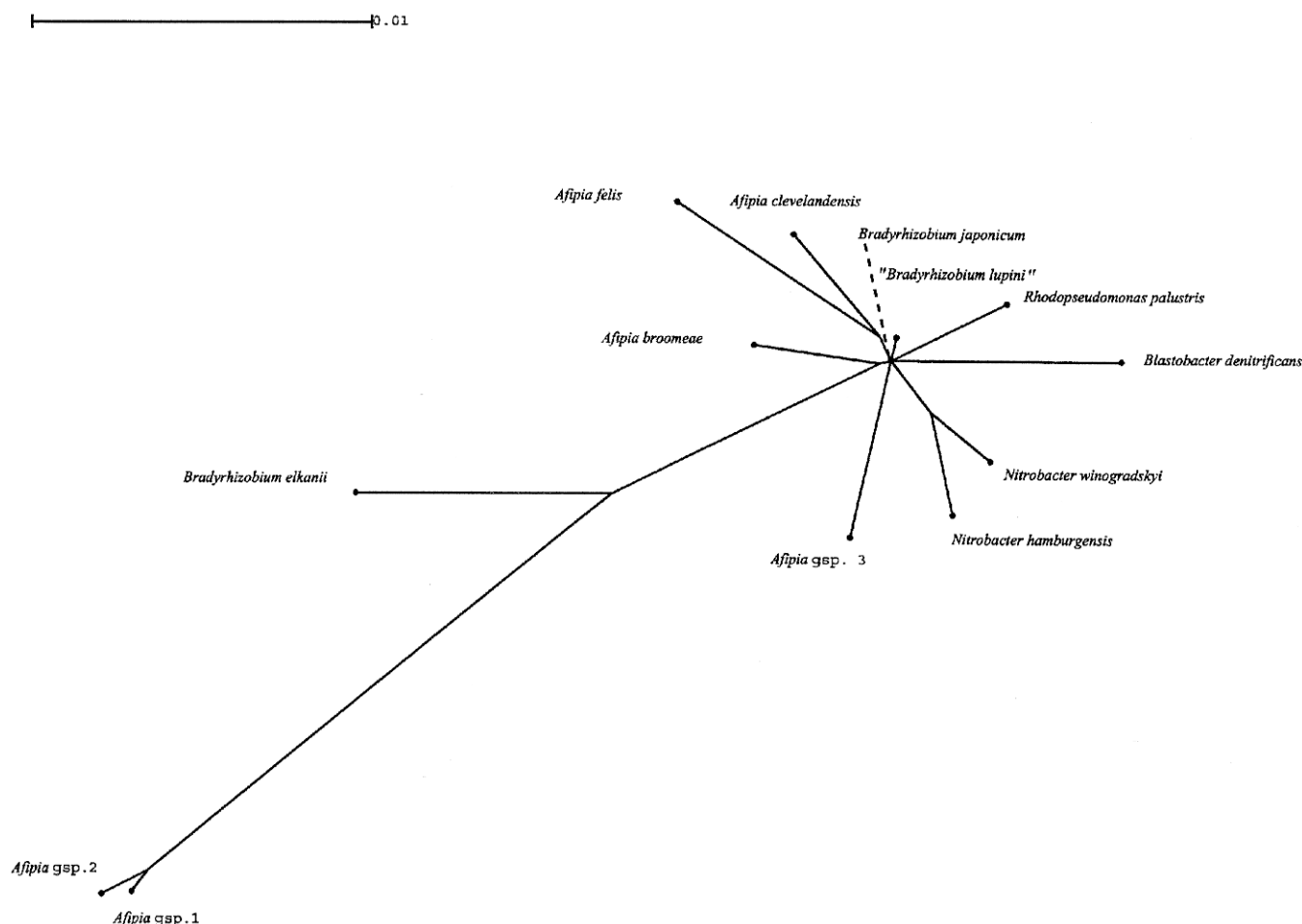


FIGURE BXII.α.172. Phylogenetic tree based on 16S rDNA sequence homologies between the type strains of *Afipia* species and type or reference strains of closely related taxa.

Genetics Mol% G + C content of the DNA ranges from 61.5 in *A. broomeae* to 69 in *Afipia* genomospecies 1 (Brenner et al., 1991a). All *A. felis* strains described by Brenner et al. (1991a) contained a 44-kb plasmid, while the other 5 species or genomospecies did not contain a plasmid.

Antibiotic sensitivity *In vitro* antibiotic sensitivity information for *Afipia* is subject to two limitations. The incubation temperature (30–32°C) differs from the standard 37°C used in the development of the National Committee for Clinical Laboratory Standards (1990); therefore, these standards cannot be used to describe resistance without further clarification. The second limitation is the small number of strains from which these data have been derived. The total published susceptibility literature for this genus consists of the original and five additional *A. felis* strains (Brenner et al., 1991a; Maurin et al., 1993), one *A. clevelandensis* strain, three *A. broomeae* strains, and one strain each of *Afipia* genomospecies 1, 2, and 3 (Brenner et al., 1991a). These studies report *A. felis* to be resistant to a wide variety of antimicrobial agents, including most beta-lactams, ciprofloxacin, and tetracycline, but susceptible to aminoglycosides and imipenem. *A. clevelandensis* and *A. broomeae* share similar susceptibility profiles with *A. felis*, except for slightly higher MICs (4–16 µg/ml) to aminoglycosides. *Afipia* genomospecies 1 and 2 are resistant to all antibiotics tested thus far including beta-lactams, ciprofloxacin, tetracycline, imipenem, and aminoglycosides. Conversely, *Afipia* genomospecies 3 is sensitive to all of the above antibiotic classes

except cefoperazone, cefoxitin, and ceftazidime. These generalizations may change as more *Afipia* strains are isolated and tested.

Pathogenicity *A. felis* has been detected in lymph nodes of patients with cat scratch disease (CSD) by culture and PCR on at least four occasions (English et al., 1988; Birkness et al., 1992; Alkan et al., 1995; Giladi et al., 1998); however, the vast majority of CSD cases appear to be caused by *Bartonella henselae*. One of the criteria for differential diagnosis of CSD is a bite or scratch from a cat. *A. felis*, however, has not been isolated from a feline source. Although English reported that antibodies made to *A. felis* reacted with specimens from CSD patients (English et al., 1988), other investigators have reported that antibody titers to *A. felis* in CSD patients were similar to titers in control groups (Amerein et al., 1996).

A. felis is capable of intracellular growth in amoebae (La Scola and Raoult, 1999a) and in tissue culture with human monocytes, macrophages, HeLa cells, and HMEC-1 cells (Birkness et al., 1992). In human macrophages *in vitro*, *A. felis* survives in phagosomes apparently by inhibiting phagosome-lysosome fusion (Brouqui and Raoult, 1993).

The isolation history of the other *Afipia* species suggests that they may act as opportunistic pathogens in humans. *A. clevelandensis* ATCC 49720 was isolated from a tibial biopsy specimen of a patient with necrotizing pancreatitis (Hall et al., 1991). *A. broomeae* ATCC 49717 was isolated from human sputum while *A.*

broomeae ATCC 49719 grew in a culture of the synovial fluid of a diabetic man with arteriosclerosis (Brenner et al., 1991a). *Afipia* genomospecies 1 ATCC 49721 was isolated from pleural fluid and *Afipia* genomospecies 2 ATCC 49722 was cultured from a bronchial lavage (Brenner et al., 1991a). *Afipia* genomospecies 3 ATCC 49723 was cultured from a water sample from Indiana, and its pathogenicity is unknown (Brenner et al., 1991a).

Ecology The majority of confirmed strains of *Afipia* have been isolated from human clinical specimens, with insufficient exposure information to identify a primary source. Multiple lines of evidence indicate that these organisms reside in fresh water. The type strain of *Afipia* genomospecies 3 was isolated from fresh water (Brenner et al., 1991a), and *A. felis* has been isolated from potable water in a hospital (La Scola and Raoult, 1999a). Many of the *in vitro* characteristics of these organisms, including their preferred growth temperature (30–32°C), lack of growth enhancement by blood or blood products, and lack of growth in elevated NaCl concentrations are also consistent with a fresh water habitat.

ENRICHMENT AND ISOLATION PROCEDURES

When incubated at 30–32°C under aerobic conditions, *Afipia* strains grow on a variety of nonselective media, including brain-heart infusion, heart infusion (with and without 5% rabbit blood), chocolate, potato dextrose, trypticase-soy (with and without 5% sheep blood), and BCYE agars. Liquid media that support *Afipia* growth include heart infusion, brain-heart infusion, and nutrient broth. Although no selective media have yet been described for these organisms, incubation of cultures at 25–30°C favors the growth of *Afipia* over many other taxa that prefer higher temperatures.

Primary isolation of *Afipia felis* strains from lymph nodes of patients with cat scratch disease is achieved by grinding tissues in equal volumes of phosphate buffered saline (pH 7.6) and sea sand, removing the sand by centrifugation at $500 \times g$ for five minutes, then centrifuging the supernatant at $10,000 \times g$ for ten minutes to obtain pelleted material. The pelleted material is then subcultured in biphasic brain-heart infusion media at 30–32°C and growth is observed between 1 and 6 days of incubation (English et al., 1988). Primary isolation is also achieved by cultivation of sonicated lymph node tissues on HeLa cell monolayers (Brenner et al., 1991a).

MAINTENANCE PROCEDURES

Long-term preservation of cultures may be achieved by suspending freshly grown cells in trypticase soy broth with 20% glycerol or defibrinated rabbit blood and freezing at -70°C or in vapor

nitrogen. For short-term maintenance (less than one year), strains should be inoculated as stabs into semisolid motility medium deeps in screw-capped tubes, incubated at 30°C until growth is observed, then placed at room temperature with the caps tightly closed (Weyant et al., 1995).

DIFFERENTIATION OF THE GENUS *AFIPIA* FROM OTHER GENERA

Other taxa that are isolated from clinical specimens and share similar phenotypic characteristics with *Afipia* are given in Table BXII.α.145. *Legionella* species, *Francisella tularensis*, and *Bordetella pertussis* may all be isolated from clinical specimens by culture on BCYE agar. The lower preferred growth temperature, the ability to grow in nutrient broth, and the hydrolysis of urea differentiates *Afipia* from these organisms. *Bartonella* species are closely related to *Afipia* phylogenetically and may also be isolated from lymph node specimens of individuals with cat-scratch disease. Unlike *Afipia*, these organisms do not grow well on BCYE agar or nutrient broth. With the exception of *B. bacilliformis*, these species prefer warmer growth temperatures and are nonmotile. The presence of C_{Br-19:1} as a predominating cellular fatty acid is also useful in differentiating *Afipia* from these other organisms.

Detailed phenotypic comparison studies including *Afipia* and its phylogenetic neighbors *Rhodopseudomonas*, *Bradyrhizobium*, *Rhizobium*, *Nitrobacter*, and *Blastobacter* have not yet been described. A limited analysis, including only type strains, indicates that *Bradyrhizobium* and *Blastobacter* acidify a wider range of carbohydrates (D-glucose, adonitol, D-galactose, D-mannose, and L-rhamnose) than *Afipia* (Weyant et al., unpublished findings). Neither the extracellular polysaccharide slime produced by *Bradyrhizobium* strains when grown on carbohydrate-containing media nor the branching or budding characteristic of *Blastobacter* cells have been observed with *Afipia* strains. *Rhodopseudomonas*, unlike *Afipia* and the other taxa in this phylogenetic cluster, is a phototroph.

FURTHER READING

- Brenner, D.J., D.G. Hollis, C.W. Moss, C.K. English, G.S. Hall, J. Vincent, J. Radosevic, K.A. Birkness, W.F. Bibb, F.D. Quinn, B. Swaminathan, R.E. Weaver, M.W. Reeves, S. O'Connor, P. Hayes, F. Tenover, A.G. Steigerwalt, B. Perkins, M.I. Daneshvar, B.C. Hill, J.A. Washington, T. Woods, S. Hunter, D.J. Wear and J. Wenger. 1991. Proposal of *Afipia*, gen. nov., with *Afipia felis*, sp. nov. (formerly the cat scratch disease bacillus), *Afipia clevelandensis*, sp. nov. (formerly the Cleveland Clinic Foundation strain), *Afipia broomeae*, sp. nov., and three unnamed genomospecies. J. Clin. Microbiol. 29: 2450–2460.
- English, C.K., D.J. Wear, A.M. Margileth, C.R. Lissner and G.P. Walsh. 1988. Cat-scratch disease: isolation and culture of the bacterial agent. JAMA (J. Am. Med. Assoc.). 259: 1347–1352.
- Müller, H.E. 1995. Investigations of culture and properties of *Afipia* spp. Zentbl. Bakteriol. 282: 18–23.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *AFIPIA*

A biochemical characterization of 6 *Afipia* species is given in Table BXII.α.146. Characteristics useful in differentiating these species are given in Table BXII.α.147. The ability to reduce nitrate is a unique characteristic that differentiates *A. felis* from the other species. Likewise, the ability to alkalize citrate is unique

to *Afipia* genomospecies 1. *A. clevelandensis* is characterized by its inability to produce acid from carbohydrates. Demonstration of specific protease activity is useful in differentiating the remaining species.

List of species of the genus *Afipia*

1. ***Afipia felis*** Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger 1992, 327^{VP} (Effective publication: Brenner, Hollis, Moss, English, Hall, Vincent,

Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger, 1991, 2458.)

fe'lis. L. gen. n. *felis* of the cat.

TABLE BXII.α.145. Differentiation of *Afipia* from similar taxa^a

Characteristic	<i>Afipia</i>	<i>Bartonella</i>	<i>Legionella</i>	<i>Francisella tularensis</i>	<i>Bordetella pertussis</i>
<i>Cellular morphology:</i>					
Straight rods	+				
Thin, slightly curved rods		+			
Thin rods			+		
Minute coccobacilli				+	+
Cell size (µm)	0.5–1.0 × 1–3	0.5–0.6 × 1–2	0.5–0.6 × 1–2	0.5–0.6 × 1–2	0.2–0.5 × 0.5–2.0
Optimal growth temperature (°C)	25–30	25–37	35–37	35–37	35–37
<i>Growth required/enhanced by:</i>					
Cysteine	+	–	+	+	–
Rabbit blood	–	+	–	+	–
Growth in nutrient broth	+	–	–	–	–
Acid production from D-glucose	–	–	–	+	–
<i>Hydrolysis of:</i>					
Gelatin	–	nd	v	–	–
Urea	+ or (+)	–	–	–	–
Nitrate reduction	v	–	–	–	–
Oxidase	+	v	+	v	+
Motility	v	v	+	–	–
<i>Predominating cellular fatty acids:</i>					
C _{10:0}				+	
C _{14:0}				+	
C _{11:0}			+		
C _{15:0} anteiso			+		
C _{16:0}		+		+	+
C _{16:1} ω7c		+			+
C _{16:0} iso			+		
C _{17:0}		+			
C _{17:0} anteiso			+		
C _{17:0} cyclo	+				
C _{18:0}	+			+	
C _{18:1} ω7c	+	+			
C _{24:1} ω15c				+	
C _{Br-19:1}	+				
C _{18:0} OH				+	

^aSymbols: +, >90% species positive; (+), delayed positive; –, <10% species positive; v, 11–89% species positive; nd, not determined; for *B. pertussis* the symbols refer to strains. Data from Brenner, et al. (1991a) and Müller (1995).

The morphological and cultural characteristics are as described for the genus. Other characteristics for this species are given in Table BXII.α.146. Rare cases of cat scratch disease have been associated with this species. The natural reservoir of this species is unknown. Most confirmed isolates have been recovered from human clinical specimens. The type strain was isolated from a lymph node of a person with cat scratch disease.

The mol% G + C of the DNA is: 62.5 (*T_m*).

Type strain: BV, ATCC 53690, CDC B91-007352, DSM 7326.

- Afipia broomeae*** Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger 1992, 327^{VP} (Effective publication: Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger, 1991, 2458.) *broomeae* a.e. N.L. gen. n. *broomeae* named after Dr. Claire V. Broome in recognition of her contributions to the epidemiology and microbiology of cat scratch disease, legionellosis, listeriosis, toxic shock syndrome, Brazilian purpuric fever, and many other diseases.

The morphological and cultural characteristics are as described for the genus. Other characteristics of the species

are given in Table BXII.α.146. The natural reservoir of this species is unknown. All confirmed strains have been isolated from human clinical material, including blood, lung tissue, and bone marrow. Presumptively pathogenic for humans. The type strain was isolated from human sputum in New Zealand in 1981.

The mol% G + C of the DNA is: 61.5 (*T_m*).

Type strain: ATCC 49717, CDC B91-007286, DSM 7327.

GenBank accession number (16S rRNA): U87759.

- Afipia clevelandensis*** Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger 1992, 327^{VP} (Effective publication: Brenner, Hollis, Moss, English, Hall, Vincent, Radosevic, Birkness, Bibb, Quinn, Swaminathan, Weaver, Reeves, O'Connor, Hayes, Tenover, Steigerwalt, Perkins, Daneshvar, Hill, Washington, Woods, Hunter, Hadfield, Ajello, Kaufmann, Wear and Wenger, 1991, 2457.) *clevelandensis* N.L. fem. adj. *clevelandensis* coming from Cleveland, Ohio, USA, where the type strain was isolated.

The morphological and cultural characteristics are as described for the genus. Other characteristics of the species are given in Table BXII.α.146. The natural reservoir of this species is unknown. The only known strain of this species was isolated from a tibial biopsy specimen of a human. Presumptively pathogenic for humans. The type strain was

TABLE BXII.α.146. Biochemical and growth characteristics of *Afipia* species^a

Characteristic	<i>A. felis</i>	<i>A. broomeae</i>	<i>A. clevelandensis</i>	<i>Afipia</i> genomospecies 1	<i>Afipia</i> genomospecies 2	<i>Afipia</i> genomospecies 3
Motility	+	+	+	+	+	+
Growth at:						
25°C	+	+	+	+	+	+
35°C	+	v	+	+	+	+
42°C	—	—	—	—	—	—
Growth on:						
MacConkey agar	v	—	—	—	—	—
<i>Salmonella</i> – <i>Shigella</i> agar	—	—	—	—	—	—
Cetrimide agar	—	—	—	—	—	—
Growth in nutrient broth	+	+	+	+	+	+
Growth in nutrient broth with 6% NaCl	—	—	—	—	—	—
Catalase	v	+	—	v	+	—
Oxidase	+	+	+	+	+	+
Alkalinization of:						
Citrate	—	—	—	+	—	—
Sodium acetate	+ or (+)	V	(+)	—	(+)	(+)
Acetamide	—	—	—	—	—	—
Serine	—	—	—	—	—	—
Tartrate	v	—	—	(+)	—	—
Litmus milk	+ or (+)	(+)	(+)	(+)	(+)	(+)
Hydrolysis of:						
Urea	+ or (+)	+ or (+)	(+)	+	+	(+)
Esculin	—	—	—	—	—	—
Gelatin	—	—	—	—	—	—
Nitrate reduction	+	—	—	—	—	—
Gas from nitrate	—	—	—	—	—	—
Nitrite reduction	—	—	—	—	—	—
Indole production	—	—	—	—	—	—
H ₂ S production (TSI butt)	—	—	—	—	—	—
Phenylalanine deaminase	+	—	—	+	—	+
L-Lysine decarboxylase	—	—	—	—	nd	—
L-Arginine dihydrolase	—	—	—	—	nd	—
L-Ornithine decarboxylase	—	—	—	—	nd	—
Acid phosphatase	+	+	+	+	+	+
Alkaline phosphatase	+	+	+	+	+	+
Phosphodiamidase	+	+	+	+	+	+
Sulfatase	+ w	+ w	+ w	+ w	+ w	+ w
Glycine aminopeptidase	+	+	+	+	+	+
L-Lysine aminopeptidase	+	+	+	+	+	+
Amylase	—	—	—	—	—	—
N-Acetyl-β-D-glucosaminidase	—	—	—	—	—	—
Galactosidase (α and β)	—	—	—	—	—	—
Glucosidase (α and β)	—	—	—	—	—	—
β-Glucuronidase	—	—	—	—	—	—
Fucosidase	—	—	—	—	—	—
Mannosidase	—	—	—	—	—	—
Xylosidase	—	—	—	—	—	—
Chymotrypsin	—	+ w	+ w	+ w	+ w	+ w
L-Phenylalanine aminopeptidase	—	+ w	+ w	+	+	+
Trypsin	—	+	—	+ w	+	+ w
L-Histidine aminopeptidase	—	+ w	—	+	+	+
L-Asparagine aminopeptidase	+	—	—	+	+	+
L-Cystine aminopeptidase	—	—	—	+	+	+
L-Isoleucine aminopeptidase	—	—	—	+	+	+
L-Leucine aminopeptidase	—	—	—	+	+	+
DL-Methionine aminopeptidase	—	—	—	+	+	+
L-Proline aminopeptidase	—	—	—	+	+	+
L-Tryptophan aminopeptidase	—	—	—	+	+	+
L-Tyrosine aminopeptidase	—	—	—	+	—	—
L-Valine aminopeptidase	—	—	—	+	—	—
Acid production from:						
D-Glucose	—	—	—	—	—	—
D-Xylose	+ w	(+)	—	(+)	(+)	(+)
D-Mannitol	—	—	—	(+)	—	—
Lactose	—	—	—	—	—	—
Sucrose	—	—	—	—	—	—
Maltose	—	—	—	—	—	—

^aSymbols are as follows: +, >90% strains positive; (+), delayed positive, may require 3–7 days incubation; +w, weak positive; —, <10% strains positive; v, 11–89% strains positive; nd, not determined. Data from Brenner, et al. (1991a) and Müller (1995).

TABLE BXII.α.147. Characteristics useful in differentiating *Afipia* species^a

Characteristic	<i>A. felis</i>	<i>A. broomeae</i>	<i>A. clevelandensis</i>	<i>Afipia</i> genomospecies 1	<i>Afipia</i> genomospecies 2	<i>Afipia</i> genomospecies 3
Growth on MacConkey agar	v	—	—	—	—	—
Catalase	v	+	—	v	+	—
Alkalinization of:						
Citrate	—	—	—	+	—	—
Sodium acetate	+ or (+)	v	(+)	—	(+)	(+)
Tartrate	v	—	—	(+)	—	—
Nitrate reduction	+	—	—	—	—	—
Phenylalanine deaminase	+	—	—	+	—	+
L-Phenylalanine aminopeptidase	—	+w	+w	+	+	+
Trypsin	—	+	—	+w	+	+w
L-Asparagine aminopeptidase	+	—	—	+	+	+
L-Tyrosine aminopeptidase	—	—	—	+	—	—
L-Valine aminopeptidase	—	—	—	+	—	—
Acid production from:						
D-Xylose	+w	(+)	—	(+)	(+)	(+)
D-Mannitol	—	—	—	(+)	—	—

^aSymbols are as follows: +, >90% species positive; (+), delayed positive, requires 3–7 days incubation; +w, weak positive; —, <10% strains positive; v, 11–89% strains positive. Data from Brenner et al. (1991a) and Müller (1995).

isolated in 1988 from a tibial biopsy specimen taken from a 69-year-old man in Ohio, USA.

The mol% G + C of the DNA is: 64 (T_m).

Type strain: ATCC 49720, CDC B91-007353, DSM 7315.

4. *Afipia* genomospecies 1

The morphological and cultural characteristics are as described for the genus. Other characteristics for the species are given in Table BXII.α.146. The natural reservoir of this species is unknown. All confirmed strains have been isolated from human clinical material. Presumptively pathogenic for humans. ATCC 49721 was isolated at Oklahoma, USA, from human pleural fluid in 1981.

The mol% G + C of the DNA is: 69 (T_m).

Deposited strain: ATCC 49721, CDC B91-007287.

5. *Afipia* genomospecies 2

The morphological and cultural characteristics are as

described for the genus. Other characteristics for the species are given in Table BXII.α.146. The natural reservoir of this species is unknown. All confirmed strains have been isolated from human clinical material. Presumptively pathogenic for humans. ATCC 49722 was isolated at Indiana, USA, from a human bronchial wash specimen in 1989.

The mol% G + C of the DNA is: 67 (T_m).

Deposited strain: ATCC 49722, CDC B91-007290.

6. *Afipia* genomospecies 3

The morphological and cultural characteristics are as described for the genus. Other characteristics for the species are given in Table BXII.α.146. The natural reservoir of this species appears to be fresh water. ATCC 49723 was isolated at Indiana, USA, from water in 1990.

The mol% G + C of the DNA is: 65.5 (T_m).

Deposited strain: ATCC 49723, CDC B91-00729.

Other Organisms

We have studied multiple clinical and non-clinical isolates that are phenotypically similar to *Afipia*, but fall into different DNA hybridization groups. These isolates have been provisionally designated *Afipia* genomospecies 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 (Weyant, unpublished findings). Phylogenetic analysis, using 16S rRNA sequence data indicate that genomospecies 4, 5, and 10 fall into the same *Rhodopseudomonas*–*Blastobacter*–*Nitrobacter*

cluster as the 6 described species. Genomospecies 11, 13, and 14 associate most closely with *Caulobacter* and *Sphingomonas*, whereas the remaining genomospecies represent novel taxa. Additional phenotypic and chemotaxonomic studies are underway to clarify the appropriate taxonomic designation of these “*Afipia*” strains.

Genus III. *Agromonas* Ohta and Hattori 1985, 223^{VP} (Effective publication: Ohta and Hattori 1983, 43)

CHRISTINA KENNEDY

Ag.ro.mon' as. Gr. n. *agros* a field; Gr. n. *monas* a unit, monad; M.L. fem. n. *Agromonas* field monad.

Bent, branched, and/or budding rods, usually 0.6–1.0 × 2–7 μm, when grown in diluted nutrient broth (NB/100). Motile by a polar flagellum. Gram negative. Colonies are colorless to white. No spores or microcysts formed. Aerobic. **Oligotrophic;** grows under conditions of low organic carbon supply (<1 mg/ml). **Fixes N₂ under microaerobic conditions. Catalase and oxidase positive.** Cellulose and starch are not hydrolyzed. Casein and gelatin are

not hydrolyzed. Cellular fatty acids consist mainly of a straight-chain unsaturated acid C_{18:1}, with smaller amounts of C_{16:0} and C_{19:1}. Ubiquinone Q-10 is present. Isolated from rice paddy soils.

The mol% G + C of the DNA is: 65.1–66.0.

Type species: *Agromonas oligotrophica* Ohta and Hattori 1985, 223 (Effective publication: Ohta and Hattori 1983, 43.)

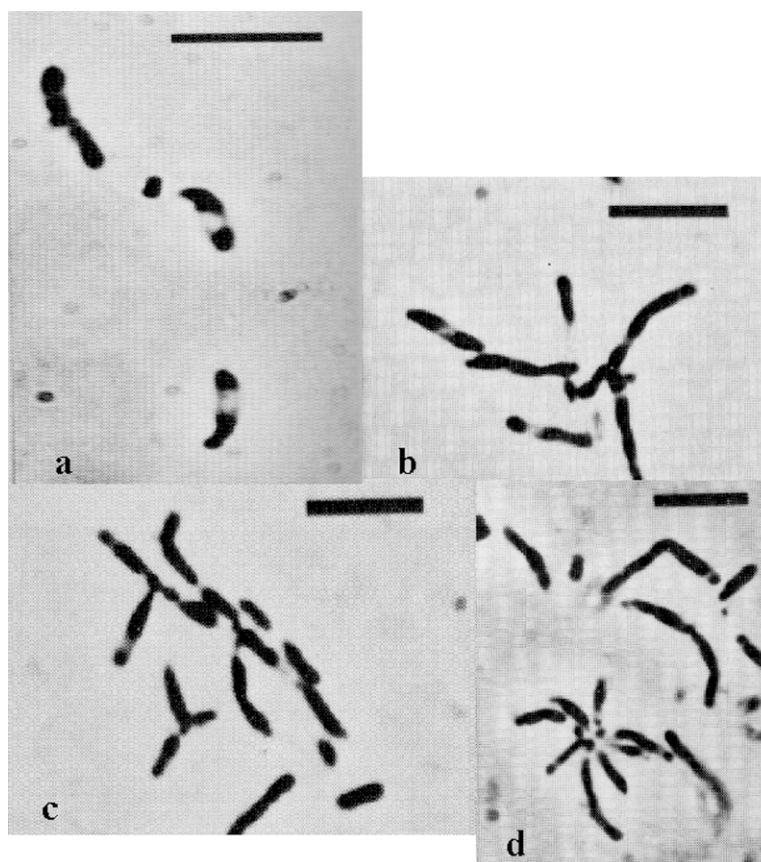


FIGURE BXII.α.173. Phase-contrast photomicrographs of 3-d-old (*a, b*) and 8-d-old (*c, d*) cultures of *A. oligotrophica* grown in NB/100 (*a*) and NB/10 (*b, c, d*) medium; (*a* and *b*) bent and budding rods in which both ends were darker than the rest of the cell; (*c*) branched rods; (*d*) a cell aggregate (rosette). Bar = 5 μm. (Reproduced with permission from H. Ohta and T. Hattori, *Antonie van Leeuwenhoek* 49: 429–446, 1983, ©Kluwer Academic Publishers, Dordrecht, Netherlands.)

FURTHER DESCRIPTIVE INFORMATION

Cell morphology Cells have an irregular rod-shaped morphology of dimensions $0.6\text{--}1.0 \times 2\text{--}7 \mu\text{m}$ (Ohta and Hattori, 1983). The irregular shape of the cells is shown in Fig. BXII.α.173. Cell division is by irregular budding and/or elongation and pinching off, not by the usual septum formation (Hattori et al., 1995).

Cell envelope Outer layers are typical for *Proteobacteria*. Electron microscopy shows a multilayered envelope structure consisting of an outer layer connected to a dark thin peptidoglycan layer, a double-layered cytoplasmic membrane, and a light space between outer and inner layers (Hattori et al., 1995) (Fig. BXII.α.174a).

Fine structure The cytoplasm is unusually divided into several compartments surrounded by the cytoplasmic membrane (Hattori et al., 1995) (Fig. BXII.α.174b). Compartmentalization develops through invagination and growth of the cytoplasmic membrane. Compartments are often connected with each other and spaces between are frequently filled with electron-dense material (Fig. BXII.α.174c). Formation of compartments is inhibited by NaCl in PM/100 medium¹ and does not occur in PM/10, although growth occurs normally in the latter medium. These

findings suggest that the developed cytoplasmic membrane and the compartmentalization of cytoplasm are not required for rapid growth but rather for growth in highly diluted nutrients. Another unusual feature is that electron-dense cores are visible in the cytoplasm, numbering from three to five. They probably represent nucleoids, as indicated by staining with the DNA-specific stain DAPI (4,6-diamidino-2-phenylindole) (Hattori et al., 1995).

Colonial or cultural characteristics: nutrition and growth Colonies are colorless/white, punctiform, pulvinate, and small (<0.5 mm diameter) (Ohta and Hattori, 1983). Growth is very sensitive to supply of organic compounds and occurs only in dilute media. Growth can occur in 1/10,000 nutrient broth (NB/10,000),² with cells reaching a density of $>10^5$ cells/ml. Growth also occurs on NB/1000, NB/100, NB/10, but not on NB. The linear relationship between the dilution of NB and cell yield indicates that growth is dependent on supplied organic carbon and is not autotrophic. Cultures reach a density of $1\text{--}5 \times 10^8$ cells/ml after 7–8 d growth on NB/100 or NB/10 (Ohta and Taniguchi, 1988a). The optimal growth temperature is 25–27°C.

Utilizable carbon sources are shown in Table BXII.α.148. The phenolic acids ferulic, *p*-coumaric, and *p*-anisic, but not benzoic,

1. Full-strength PM medium consists (g/l): peptone, 10.0; and meat extract, 10.0.

2. Full-strength NB contains (g/l of tap water): peptone, 10.0 g; meat extract, 10.0 g; and NaCl, 5.0 g; pH 7.0–7.2.

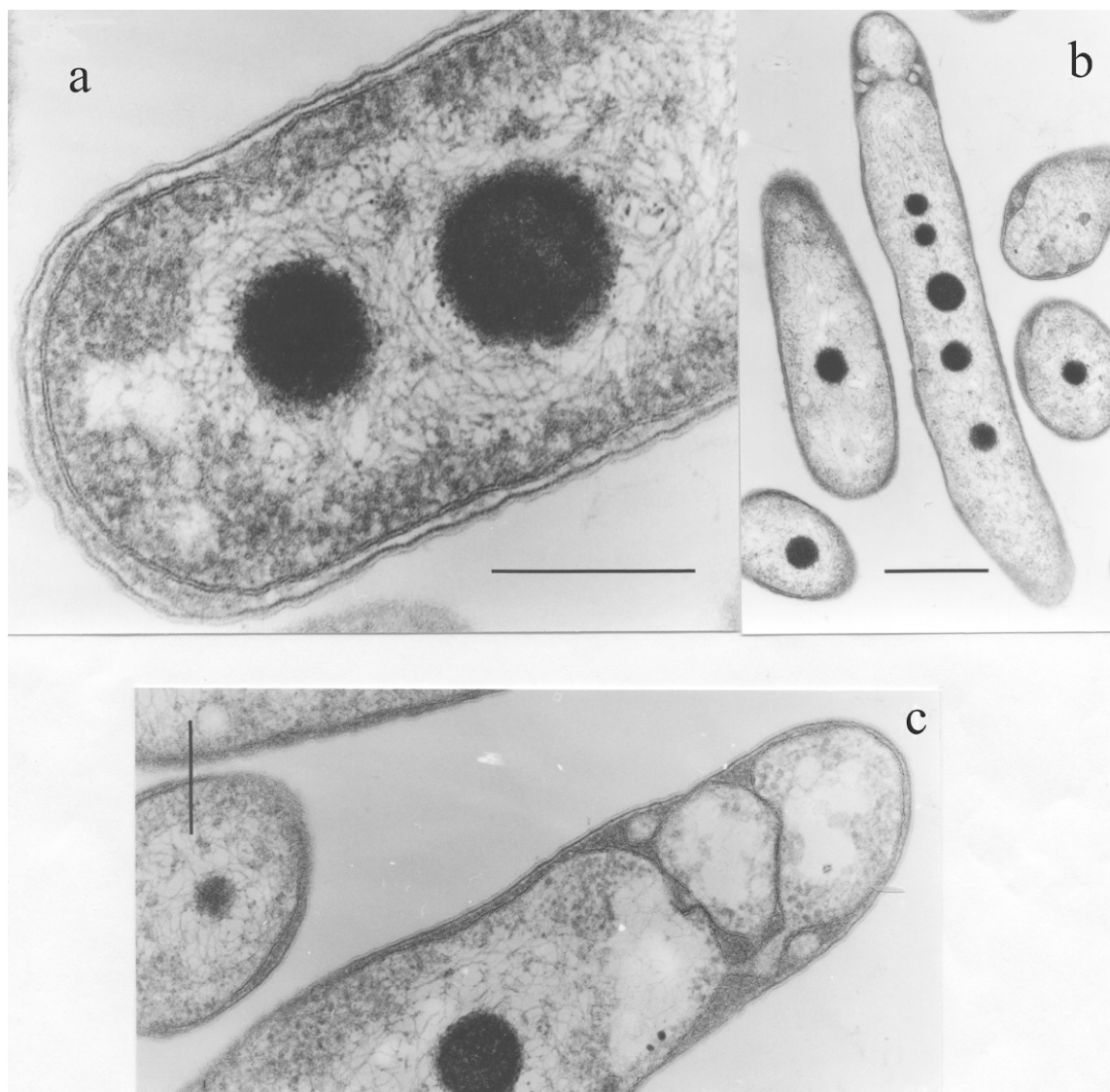


FIGURE BXII.α.174. Electron micrograph of *Agromonas oligotrophica* grown in PM/100. Sections were stained with 2% (w/v) aqueous uranyl acetate and saturated lead citrate. (a) The multilayered structure of cell envelope and the nucleoid-like structures in cytoplasm. Bar = 0.2 μ m. (b) Growing cells; note that the cytoplasm is divided into compartments with electron-dense cores in nuclear zones. Bar = 1 μ m. (c) Compartments can be separate or connected. Bar = 0.2 μ m. (Reproduced with permission from T. Hattori et al., *Journal of General and Applied Microbiology* 41: 23–30, 1995, ©Microbiology Research Foundation, Tokyo, Japan.)

support growth. Ferulic acid degradation requires organic nutrients to be present in the growth medium. The pathway includes conversion of ferulic acid to vanillate and then probably to protocatechuate (Ohta, 2000).

Growth occurs microaerobically on nitrogen-free medium (NF).³ On NF semi-solid medium, cells grow at a depth of 18–25 mm below the surface. On media supplemented with 50 mg/l $(\text{NH}_4)_2\text{SO}_4$, growth occurs at a depth of 8–12 mm (Ohta and Hattori, 1983), indicating use of ammonium as the N source at a higher $p\text{O}_2$ than for utilization of N_2 . Acetylene reduction occurs, verifying nitrogen fixation by *A. oligotrophica*; the specific

activity is ~ 10 nmoles/min/mg protein, similar to the activity in many other free-living diazotrophs. Nitrate is reduced to nitrite.

Metabolism and metabolic pathways *A. oligotrophica* is an aerobic/microaerobic organism not extensively characterized in terms of metabolic features. In one study of respiratory characteristics, growth of *A. oligotrophica* on 1% trypticase peptone plus 0.1% yeast extract was biphasic (Ohta and Taniguchi, 1988b). The molar growth yield on oxygen consumed in the first phase increased 1.7-fold in the second phase. Cytochromes of the *a*, *b*, and *c* type are present, as well as a CO-binding *b*-type cytochrome, thought to be cytochrome *o*. The pattern was not detectably changed by medium dilution.

Ecology Strains of *Agromonas oligotrophica* have been isolated from rice paddy soils. They may play a role in decomposing organic matter such as the phenolic acids, ferulic, and coumaric,

3. NF medium contains (per liter of distilled water): K_2HPO_4 , 50 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; NaCl , 5 mg; CaCl_2 , 0.1 mg; FeCl_3 , 0.1 mg; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 mg; 2-oxoglutaric acid, 0.3 or 1 g; pH 6.8–7.5. The carbon source—2-oxoglutaric acid—is sterilized by filtration and added aseptically to the autoclaved medium.

TABLE BXII.α.148. Characteristics of *Agromonas oligotrophica*^a

Characteristic	Reaction
<i>Growth at:</i>	
4°C	—
10–37°C	+
42°C	d
Hydrolysis of gelatin, casein, starch, and cellulose	—
Fluorescent pigment production	+
Polyhydroxybutyrate production	+
Catalase, oxidase	+
<i>Growth on:</i>	
NB	—
NB/10–NB/10,000	+
<i>Utilization of:</i>	
Glucose, galactose, mannose, xylose, L-arabinose, acetic acid, lactic acid, gluconate, pyruvate, citrate, 2-oxo-glutarate, succinate, L-malate, ferulic acid, <i>p</i> -coumaric acid, <i>p</i> -anisic acid	+
Cellobiose, maltose, lactose, raffinose, benzoate, methanol	—

^aSymbols: see standard definitions.

and recycling other nutrients in environments low in organic substrates. Nitrogen fixation may be of ecological significance, allowing occupation of this niche.

Ohta and Hattori (1983) reported that irregular rod-shaped, oligotrophic bacteria, many of which were identical to *A. oligotrophica*, were abundant in the roots of rice plants numbering 10^8 – 10^9 cells/g dry matter. Whether fixed nitrogen can be provided for plant nutrition is not known.

Fatty acid composition The predominant fatty acid in five strains tested is an 18-carbon straight-chain unsaturated acid with one double bond (C_{18:1}) (Ohta and Hattori, 1983). Present also among the isolates was a C_{16:0} fatty acid (at 9–14% of total) and C_{19:1} fatty acid (6–18% of total). Ubiquinone Q-10 was found in all isolates.

ENRICHMENT AND ISOLATION PROCEDURES

Oligotrophic bacteria are widely distributed in nature and share the ability to grow on low nutrient media. At microsites of soil, selective growth of oligotrophic bacteria occurs at higher probabilities with lower concentrations of nutrients and at lower probabilities with higher concentrations of nutrients (Hattori, 1981). Consequently, the isolation of *Agromonas* is based on this feature. Soil suspensions are diluted 10^{-3} and 10^{-4} ; 1 ml of each dilution is plated onto NB/100 medium containing 1% agar. After 1 week to 1 month, colonies are tested for sensitivity to nutrients by plating on 1 × NB. NB-sensitive colonies are then examined for other characteristics as described.

MAINTENANCE PROCEDURES

The medium for maintenance is NB/100 containing 0.3–0.4% agar, dispensed in screw-cap tubes. Cells are kept as a stabbed culture at room temperature. Cells remain viable for several years; transfer every 3–4 years is recommended. An alternative method is the suspension of cells in 20% glycerol followed by storage at –80°C. Cells stored this way are less viable in the long

term than those described above (H. Ohta, personal communication).

DIFFERENTIATION OF THE GENUS *AGROMONAS* FROM OTHER GENERA

The organisms most closely related to *Agromonas*, as indicated by 16S rDNA analysis, are species of *Blastobacter*, *Bradyrhizobium*, and the unclassified DNB strains described by Saito et al. (1998) (see Taxonomic Comments). Table BXII.α.149 summarizes the differential features of these organisms.

TAXONOMIC COMMENTS

Based on comparison of morphological and physiological characteristics, Ohta and Hattori (1983) placed five paddy soil isolates in a new genus, *Agromonas*, and a new species, *Agromonas oligotrophica*. The morphological and physiological characteristics were most similar to those of the genera *Xanthobacter* and *Azospirillum*, but 16S rDNA analysis (Saito et al., 1998) indicated a close relationship between *Agromonas oligotrophica* and *Blastobacter denitrificans*, which form a group (Fig. BXII.α.175). *B. denitrificans* does not fix nitrogen and, unlike *A. oligotrophica*, it can utilize methanol and denitrify (Hirsch and Müller, 1985). However, *B. denitrificans* is oligotrophic and the rod-shaped cells are bent and budding like those of *A. oligotrophica*. Other oligotrophs, called DNB (dilute nutrient broth) bacteria, are also placed in this group (Saito et al., 1998). These DNB strains were isolated from grassland soil by Ohta and Hattori (1980), and only 1 of 11 was able to fix N₂. The DNB isolates were similar to each other in a number of ways but were not assigned a genus or species name. The group containing *B. denitrificans*, *A. oligotrophica*, and the unclassified DNB bacteria is most closely related to a *Bradyrhizobium* sp. Unlike the latter species, neither *A. oligotrophica* nor the DNB bacteria can nodulate siratro (*Macroptilium atropurpureum*). In DNA–DNA hybridization tests, the DNB bacteria appeared more closely related to the *Bradyrhizobium* sp. than to *A. oligotrophica*. The taxonomic cluster carrying *A. oligotrophica*, *B. denitrificans*, the DNB strains, and the *Bradyrhizobium* sp. is related most closely with a group composed of *Rhodopseudomonas palustris*, two *Nitrobacter* species, and the genus *Afipia* (together named the BANA domain; Saito et al., 1998). *Agromonas oligotrophica* is a member of the phylum *Proteobacteria*, order *Rhizobiales*, and the family *Bradyrhizobiaceae*.

ACKNOWLEDGMENTS

I am grateful to Drs. T. Hattori, K. Minamisawa, R. Hattori, and H. Ohta for their cooperation in providing clarifying information, reprints, and electron micrographs, and for their helpful comments concerning the manuscript. Their work allowed *Agromonas oligotrophica* to be known to the world.

FURTHER READING

- Hattori, T. and R. Hattori. 2000. The plate count method: an attempt to delineate the bacterial life in the microhabitat of soil. In Bollag and Stotzky (Editors), *Soil Biochemistry*, Vol. 10, Marcel Dekker Inc., New York, NY. pp. 271–302.
- Saito, A., H. Mitsui, R. Hattori, K. Minamisawa and T. Hattori. 1998. Slow-growing and oligotrophic soil bacteria phylogenetically close to *Bradyrhizobium japonicum*. *FEMS Microbiol. Ecol.* 25: 277–286.

List of species of the genus *Agromonas*

1. ***Agromonas oligotrophica*** Ohta and Hattori 1985, 223^{VP} (Effective publication: Ohta and Hattori 1983, 43.)
ol.i.go.tro'phi.ca. Gr. adj. *oligo* few, low; Gr. n. *tropheia* nourishment; M.L. adj. *oligotrophica* low nutrients.

The characteristics are as described for the genus and in Tables BXII.α.148 and BXII.α.149. Additional characteristics are as follows. Several cells may adhere to each

TABLE BXII.α.149. Characteristics distinguishing *Agromonas oligotrophica* from related taxa^a

Characteristic	<i>Agromonas oligotrophica</i>	DNB bacteria ^b	<i>Blastobacter denitrificans</i>	<i>Bradyrhizobium japonicum</i> (USDA Strain 110)	<i>Azospirillum brasilense</i>	<i>Xanthobacter</i> sp.
Budding	+	+	+	—	—	+
Flagellar arrangement:						
Polar	+	—	—	+	+	—
Subpolar	—	+	+	—	+	—
Acid from glucose	—	—	+	—	+	—
Denitrification	—	—	+	—	d	—
Methanol utilization	—	—	+	—	—	—
Nodulation of siratro	—	—	—	+	—	—
N ₂ fixation in culture	+	—	—	± ^c	+	+

^aSymbols: see standard definitions.
^bDilute nutrient broth.
^cLow activity, only in certain media (Keister, 1975).

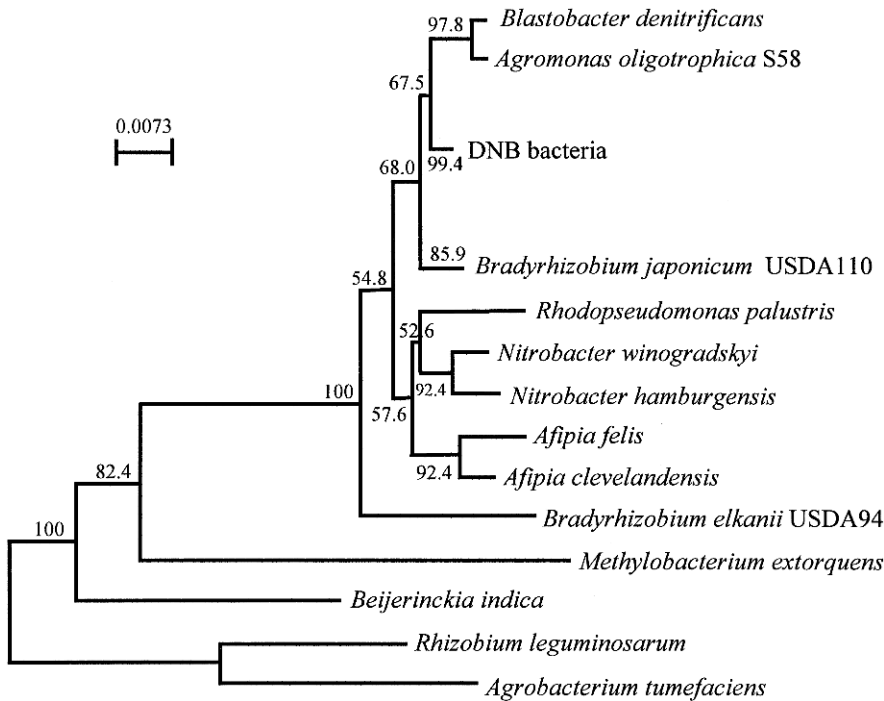


FIGURE BXII.α.175. Phylogenetic relationships of *Agromonas oligotrophica* to other members of the Alphaproteobacteria based on near full-length sequence similarities of 16S rRNA genes. The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap values are shown at nodes. The scale bar indicates substitutions per site. The group DNB are unclassified oligotrophs, mostly non-nitrogen fixing, isolated from grassland soil (Saito et al., 1998).

other, forming a rosette. Colonies are colorless/white, punctiform, pulvinate, and entire.

NaCl, KCl, Casamino acids, peptone, and meat extract inhibit growth at 0.5–1.0%. Several sugars and organic acids are utilized. The aromatic acids ferulic, *p*-coumaric, and *p*-anisic can be utilized, but not benzoic acid. Neither acid

nor gas is produced from glucose. Cellulose and starch are not hydrolyzed.

The mol% G + C of the DNA is: 65.1–66.0 (*T_m*).

Type strain: ATCC 43045, DSM 12412, JCM1494.

GenBank accession number (16S rRNA): D78366.

Genus IV. *Blastobacter* Zavarzin 1961, 962^{AL} emend. Sly 1985, 44

LINDSAY I. SLY AND PHILIP HUGENHOLTZ

Blas.to.bac' ter. Gr. n. *blastos* bud, shoot; M.L. n. *bacter* equivalent of Gr. masc. n. *bactrum* rod; M.L. masc. n. *Blastobacter* a budding rod.

Cells ovoid rods, wedge or club shaped, or pleomorphic, often slightly curved and occasionally branched. Cell poles are rounded or slightly tapering on one pole. Cell size range is 0.5–1.0 × 1.0–

4.5 μm. Cells may form rosettes. Gram negative. New cell formation and multiplication occur by budding on the free cell pole, subpolarly or laterally. Young buds initially rod shaped, ovoid,

or spherical to oblong. Buds may be released or remain attached. **Motile, flagellated swarmer cells may occur** in some species. Do not form spores or cysts. Some strains produce large amounts of exopolymer. Grow in liquids as turbidity, pellicle, or precipitate. Colony pigmentation may be yellow, or colorless, becoming brown in older cultures.

Aerobic. Heterotrophic. Oxidase, catalase, and peroxidase positive. Some species grow chemolithotrophically with hydrogen. Some species may fix CO₂ reductively when grown on methanol or methylated amines. Optimal pH between 6.8 and 7.8. Temperature range for growth is 10–46°C. Carbon and energy sources may be alcohols, sugars, organic acids, or some amino acids. Ammonium, nitrate, urea, peptone, yeast extract, or casein hydrolysate may be utilized as nitrogen sources. May reduce nitrate to nitrite, or denitrify.

Isolated from freshwater lakes, ponds, and groundwater, as well as activated sludge.

The mol% G + C of the DNA is: 59–69.

Type species: *Blastobacter henricii* Zavarzin 1961, 962.

FURTHER DESCRIPTIVE INFORMATION

Cells of *Blastobacter* generally have a rod-shaped morphology and reproduce by budding at the poles or laterally, with cells often attaching at the nonreproductive pole to form rosettes (Fig. BXII.α.176). The characteristics of the species of *Blastobacter* are mainly based on the study of the type strains of the five species and little is known of the diversity within each species. All strains have yet to be characterized over a constant range of tests in a single polyphasic study, making it difficult at this time to compare the species. A continuing difficulty is the absence of a culture for the type species *B. henricii*, whose description is based solely on morphological characteristics obtained from examination of an enrichment culture (Zavarzin, 1961). 16S rRNA sequences are available for the three validly published species *B. aggregatus*, *B. capsulatus*, and *B. denitrificans*, but not for the two species "*B. aminooxidans*" and "*B. viscosus*" that have not been validated.

Hirsch and co-workers have searched for *Blastobacter* cultures from various freshwater habitats and obtained pure cultures from lakes and ponds (Hirsch and Müller, 1985), resulting in the description of three new species—*B. aggregatus*, *B. capsulatus*, and *B. denitrificans*—which together with *B. henricii* are the only validly described species. In the absence of a type culture for *B. henricii*,

Hirsch and Müller (1985) argued that their new species belonged to the genus *Blastobacter* based on cellular morphological similarity, and physiological differences with other budding bacteria such as *Rhodopseudomonas*, *Nitrobacter*, and *Methylosinus trichosporium*.

Morphologically, cells of *B. aggregatus*, *B. capsulatus*, and *B. denitrificans* exhibit budding, and no cross-septation occurs without prior budding (Hirsch and Müller, 1985). Cells of these species are essentially rod shaped and of varying length; shorter cells are often ovoid. Cells of *B. capsulatus* IFAM 1004 have a tendency to bend or even twist helically (Hirsch and Müller, 1985). Of these, only *B. aggregatus* IFAM 1003 forms rosettes, and is therefore most morphologically similar to the description of the type species *B. henricii*. Two species, *B. aggregatus* and *B. denitrificans*, produce motile swarmer cells with flagella while *B. capsulatus* and *B. henricii* are nonmotile.

B. aggregatus, *B. capsulatus*, and *B. denitrificans* utilize D-glucose, D-fructose, lactose, D-ribose, D-galactose, mannitol, glycerol, pyruvate, α-oxoglutarate, acetate, L-glutamate, and L-histidine as carbon compounds, but are unable to utilize maltose, starch, dextrin, inulin, fumarate, lactate, caproate, citrate, propionate, indole, phthalate, D,L-phenylalanine, L-aspartate, leucine, L-serine, glycine, and alanine. These species are reported to produce acid but no gas anaerobically from D-fructose, and D-galactose in the Hugh and Leifson fermentation test (Hirsch and Müller, 1985). Production of acid aerobically from D-glucose and mannitol varies among species (Table BXII.α.150). In addition to the generic characters, these species do not decarboxylate arginine or lysine, deaminate lysine, or phenylalanine, hydrolyze gelatin or cellulose, or produce acetoin, indole, or H₂S. Ammonium, nitrate, and methane are not oxidized. Litmus milk is not acidified, peptonized, or coagulated (Hirsch and Müller, 1985).

Two nomenclaturally invalid species, "*B. aminooxidans*" and "*B. viscosus*", were isolated from activated sludge and studied extensively for their morphological and physiological characteristics (Loginova and Trotsenko, 1979; Doronina et al., 1983). These species grow on C₁ compounds as carbon and energy sources and are facultative autotrophs capable of chemolithotrophic growth with hydrogen. Strains of "*B. viscosus*" (Loginova and Trotsenko, 1979) were isolated from activated sludge of the purifying installation of the Baikal Pulp and Paper Combine in Russia. Studies on the formation of the bud in "*B. viscosus*" show that budding begins with a centripetal ingrowth of the plasma membrane and the cell wall with full delimitation of the bud from the maternal cell. Incomplete separation and multiple budding leads to the formation of pleomorphic cells. Crystalloid formations consisting of parallel arrangements of electron dense and electron transparent filamentous structures are observed in the region of bud formation (Loginova and Trotsenko, 1979).

When grown on a medium with methanol as carbon source, "*B. viscosus*" produces a heteropolysaccharide consisting of galactose, glucose, rhamnose, xylose, and glucuronic acid (Loginova and Trotsenko, 1979).

"*B. viscosus*" grows weakly with methylamine, dimethylamine, formate, formamide, and dimethylformamide, but not trimethylamine, formaldehyde, dimethyl sulfoxide, or dimethylsulfone as carbon sources (Loginova and Trotsenko, 1979).

Ribulose phosphate carboxylase is induced in "*B. viscosus*" when C₁ compounds are utilized, but no activities of specific



FIGURE BXII.α.176. Photomicrograph of actively growing culture of *Blastobacter aggregatus* on PYG medium, showing budding, rod-shaped cells, and rosette formation. Bar = 10 μm.

*Editorial Note: Recently "*Blastobacter viscosus*" and "*Blastobacter aminooxidans*" have been reclassified as *Xanthobacter viscosus* and *Xanthobacter aminooxidans* (Doronina and Trotsenko, 2003).

TABLE BXII.α.150. Differential characteristics of the genus *Blastobacter* species and other heterotrophic, Gram-negative, rod-shaped budding bacteria^a

Characteristic	<i>Blastobacter henrici</i>	<i>Blastobacter aggregatus</i>	<i>Blastobacter capsulatus</i>	<i>Blastobacter denitrificans</i>	" <i>Blastobacter aminooxidans</i> "	" <i>Blastobacter viscosus</i> "	<i>Blastomonas natatoria</i>	<i>Agromonas oligotrophica</i>	<i>Gemmibacter aquatilis</i>
<i>Cellular morphology:</i>									
Shape	Rods, wedge- or club-shaped, often curved	Ovoid to rod-shaped	Rods, often bent and tapering on budding pole, older cells Y-shaped	Rods with rounded poles	Pleomorphic rods with minute appendages	Pleomorphic rods, often bent and branched	Rods, slightly curved, or wedge-shaped	Bent, branched rods	Ovoid to rod-shaped, short chains
Cell length (μm)	2.0–4.5	1.5–2.3	1.5–2.3	1.5–2.3	1.5–3.0	1.0–3.2	1.0–3.0	2.0–7.0	1.2–2.7
Cell width (μm)	0.7–1.0	0.6–0.8	0.7–0.9	0.6–0.8	0.8–1.0	0.5–0.9	0.5–0.8	0.6–1.0	1.0–1.2
<i>Initial bud shape:</i>									
Ovoid			+		+	+			
Rods		+		+				+	+
Spherical							+		
Spherical or ovoid									
Spherical or oblong	+								
Bud origin	Polar	Narrow pole	Narrow pole or lateral	Slightly subpolar	Polar or lateral	Polar or subpolar	Polar	Polar	Polar or subpolar
Capsule formation	nd ^b	–	+	–	+	+	–	nd	–
Motility	–	+	–	+	–	–	+	+	–
Rosette formation	+	+	–	–	–	–	+	+	–
Colony pigmentation	nd	Colorless, slightly brownish when older	Colorless	Colorless, brownish when older	Yellow (orange)	Yellow	Yellow or pale pink	Colorless	Colorless
Origin	Forest brook water	Lake water	Eutrophic pond water	Lake water	Activated sludge	Activated sludge	Swimming pool	Paddy soil	Forest pond
Mol% G + C	nd	60	59	65	69	66	65	66	63

(continued)

TABLE BXII.α.150. (cont.)

Characteristic	<i>Blastobacter henricii</i>	<i>Blastobacter aggregatus</i>	<i>Blastobacter capsulatus</i>	<i>Blastobacter denitrificans</i>	" <i>Blastobacter aminooxidans</i> "	" <i>Blastobacter viscosus</i> "	<i>Blastomonas natatoria</i>	<i>Agromonas oligotrophica</i>	<i>Gemmibacter aquatilis</i>
<i>Utilization of carbon sources:</i>									
Sucrose	nd	+	+	-	+	+	nd	nd	+
Cellobiose	nd	+	+	-	+	+	nd	-	nd
Salicin	nd	-	+	-	nd	nd	nd	nd	nd
Glucuronic acid lactone	nd	-	-	+	nd	nd	nd	nd	nd
N-Acetyl-glucosamine	nd	-	-	+	+	+	nd	nd	-
Succinate, malate	nd	-	-	+	+	+	nd	+	+
Formate, formamide	nd	-	-	+	+	+	nd	nd	nd
Tartrate	nd	-	-	+	nd	-	nd	nd	+
Glutamate	nd	nd	nd	nd	nd	nd	nd	nd	+
L-Arginine	nd	+	-	-	-	-	nd	nd	+
L-Proline	nd	+	+	-	nd	-	nd	nd	nd
Methanol	nd	-	-	+	-	+	-	-	-
Ethanol	nd	+	-	+	+	+	nd	nd	+
<i>Acid production from:</i>									
Glucose	nd	+	+	-	-	+	+	-	+
Mannitol	nd	-	+	-	nd	nd	-	nd	+
Utilization of urea as N source	nd	+	-	+	nd	-			
<i>Nitrate reduction:</i>									
Assimilatory	nd	-	+	-	+	+	-	nd	+
Dissimilatory	nd	-	+	+	+	+	-	nd	+
Hydrogen autotrophy	nd	nd	nd	nd	+	+	-	nd	-
Temperature range for growth (°C)	nd	13-43	14-35	13-46	10-34	10-34	11-39	nd	17-39
Temperature optimum (°C)	nd	36	27	41	29-32	28-30	25-30	nd	31
pH optimum for growth	nd	6.9	7.3-7.8	6.8-7.2	7.2-7.8	6.8-7.2	nd	nd	nd

^aData from Loginova and Trotsenko (1979), Doronina et al. (1983), Hirsch and Müller (1985), Rothe et al. (1987), Trotsenko et al. (1989), and Saito et al. (1998).

^bnd, not determined.

enzymes of the serine and hexulose monophosphate pathways characteristic of methylotrophs were observed. "*B. viscosus*" is apparently able to utilize the carbon from methanol autotrophically after its oxidation to CO₂ (Loginova and Trotsenko, 1979).

Like "*B. viscosus*", "*B. aminooxidans*" was also isolated from activated sludge. "*B. aminooxidans*" was the first organism in which autotrophic assimilation of methylated amines was shown. As well as the ability to utilize methylated amines, "*B. aminooxidans*" is able to grow chemolithotrophically with hydrogen (Doronina et al., 1983). Trimethylamine is oxidized to dimethylamine and formaldehyde by dehydrogenase (phenazine methosulfate). Methylamine is further oxidized to formaldehyde and ammonia, and the formaldehyde is oxidized via formate to CO₂. The CO₂ is assimilated by the ribulose biphosphate pathway. The serine and hexose monophosphate pathways are not present. The cells possess enzymes of the tricarboxylic acid cycle and the glyoxylate shunt. Ammonium is assimilated via reductive amination of α -glutarate, pyruvate, and glyoxylate, and via the glutamate cycle (Doronina et al., 1983).

In "*B. aminooxidans*", trimethylamine and methylamine are oxidized by a monooxygenase (NADH or NADPH). Formaldehyde is oxidized via formate to CO₂ by dehydrogenases: the CO₂ is refixed into 3-phosphoglyceric acid by using the autotrophic ribulose-1,5-bisphosphate pathway (Trotsenko et al., 1989). Phosphoribulose kinase is also present and active. In "*B. viscosus*" autotrophic growth occurs in an atmosphere of H₂/O₂/CO₂ or with methanol. In both cases, cells assimilate CO₂ via the ribulose biphosphate pathway and show active phosphoribulokinase (PRK) and ribulose biphosphate carboxylase (RBPC). In contrast to PRK, the RBPC is completely repressed in glucose-grown cells. The primary CO₂ acceptor is regenerated by transaldolase and transketolase activity (Trotsenko et al., 1989).

Cells of "*B. viscosus*" possess dehydrogenase activity, catalyzing methanol oxidation via formaldehyde and formate to CO₂. The serine and hexulose-phosphate pathways of C₁ metabolism do not operate due to absence of hydroxypyruvate reductase, serine-glyoxylate aminotransferase, ATP malate lyase, and hexulose-phosphate synthase. Fructose-1,6-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase (NAD) play an important role in metabolic conversions of phosphotrioses. The cells contain all enzymes of the citric acid cycle with lower levels in methanol-grown cells than in glucose-grown cells. C₄ compounds are resynthesized mainly by carboxylation of pyruvate and phosphoenolpyruvate (Trotsenko et al., 1989).

ENRICHMENT AND ISOLATION PROCEDURES

A low-nutrient medium containing glucose, peptone, and yeast extract such as that described by Staley (1981b) is suitable for the enrichment and isolation of *Blastobacter*. Cultures may be isolated by direct plating of environmental samples, or after enrichment. Alternatively, water samples may be enriched with 0.005% peptone and incubated aerobically at 18–23°C for 1–3 weeks (Hirsch and Müller, 1985; Trotsenko et al., 1989). "*Blastobacter viscosus*" was isolated from activated sludge after enrichment in mineral salts medium containing 1% methanol at 30°C for 7 d (Loginova and Trotsenko, 1979).

MAINTENANCE PROCEDURES

Cultures of *B. aggregatus*, *B. capsulatus*, and *B. denitrificans* are reported (Hirsch and Müller, 1985) to grow well on dilute peptone–yeast extract–glucose (PYG) medium (Staley, 1981b). "*B. viscosus*" grows well on media with methanol as the carbon source

(Loginova and Trotsenko, 1979), and "*B. aminooxidans*" may be grown on media with trimethylamine as the carbon source or on glucose potato agar (Doronina et al., 1983). Cultures may be preserved by cryogenic storage in liquid nitrogen when suspended in sucrose peptone broth containing 10% glycerol, and by freeze-drying in glucose peptone broth containing horse serum.

DIFFERENTIATION OF THE GENUS *BLASTOBACTER* FROM OTHER GENERA

Budding of bacteria is widespread throughout the *Proteobacteria* and occurs in phylogenetically diverse genera with widely different physiological characteristics (Rothe et al., 1987; Hugenholtz et al., 1994; Sly and Cahill, 1997) including *Agromonas*, *Blastobacter*, *Blastomonas*, *Gemmobacter*, *Rhodopseudomonas*, *Methylosinus*, and *Nitrobacter*. Table BXII.α.150 gives the characteristics that are useful in differentiating the species of Gram-negative, heterotrophic rod-shaped budding bacteria.

TAXONOMIC COMMENTS

The taxonomy of the genus *Blastobacter* Zavarzin 1961 is in need of revision. The polyphyletic nature of the genus is well established (Hugenholtz et al., 1994; Sly and Cahill, 1997), and emphasizes that morphological characteristics such as budding cell division, which define the genus, are not phylogenetically useful features at the genus level and may group distantly related species in the genus as currently defined (Zavarzin, 1961; Sly, 1985; Trotsenko et al., 1989). Cell division by nonprosthecae budding is confined to the *Alphaproteobacteria* but is widely distributed in physiologically diverse genera within the class. As reported previously (Hugenholtz et al., 1994; Sly and Cahill, 1997), resolution of the taxonomic confusion is impeded by the lack of a type strain for the type species, *Blastobacter henricii* (Zavarzin, 1961; Skerman et al., 1989), which was never obtained in pure culture.

The genus *Blastobacter* was proposed by Zavarzin (1961) to include rosette-forming, budding, rod-shaped or wedge-shaped bacteria that were observed in a filter paper enrichment of reduced iron-containing water from a northern Russian forest brook. Zavarzin was unable to isolate the cells in pure culture, and the description of *Blastobacter henricii* is based on drawings and observations.

Several additional *Blastobacter* species have been validly described since 1961 (Moore and Moore, 1992). These include *Blastobacter aggregatus*, *Blastobacter capsulatus*, *Blastobacter denitrificans* (Hirsch and Müller, 1985), and *Blastobacter natatorius* (Sly and Hargreaves, 1984; Sly, 1985). *Blastobacter natatorius* was later transferred and became the type species of the genus *Blastomonas* in the first step to clarify the taxonomy of the genus *Blastobacter* (Sly and Cahill, 1997). Other taxonomically invalid species include "*Blastobacter aminooxidans*" (Doronina et al., 1983), "*Blastobacter viscosus*" (Loginova and Trotsenko, 1979), and "*Blastobacter novus*" (Rezanka et al., 1991). Several authors have demonstrated that there is a high degree of heterogeneity in the genus *Blastobacter* with respect to phenotype (Trotsenko et al., 1989), cellular fatty acids and phospholipids (Sittig and Hirsch, 1992), and molecular phylogeny (Rothe et al., 1987; Green and Gillis, 1989; Hugenholtz et al., 1994; Sly and Cahill, 1997; Willems et al., 2001).

Fig. BXII.α.177 shows the phylogenetic relationships of the species of *Blastobacter* for which 16S rRNA sequences are available. An analysis of the 16S rRNA sequences of the validated species shows that species of *Blastobacter* are polyphyletic and belong to

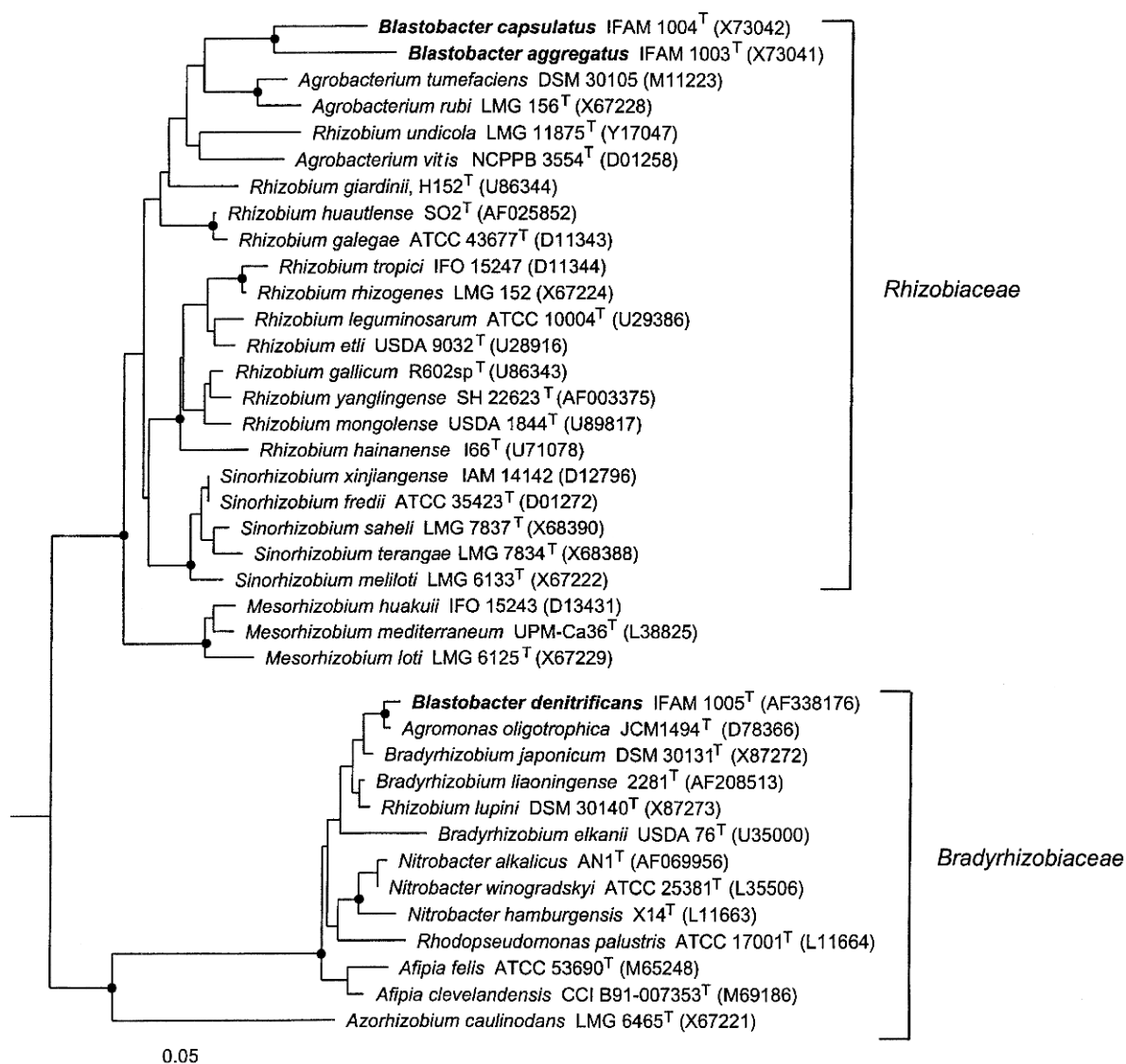


FIGURE BXII.α.177. Phylogenetic tree showing the polyphyletic positions of *Blastobacter* species in the *Alphaproteobacteria* based on 16S rRNA sequence similarities.

separate lineages in the *Alphaproteobacteria*. *Blastobacter aggregatus* and *Blastobacter capsulatus* are close phylogenetic relatives belonging to a strongly supported branch of the family *Rhizobiaceae*, whereas *Blastobacter denitrificans* belongs to a cluster containing the genus *Bradyrhizobium* and *Agromonas oligotrophica* in the family *Bradyrhizobiaceae*. *Blastobacter aggregatus* and *Blastobacter capsulatus* are closely related to each other (96.6% sequence similarity) and to *Agrobacterium tumefaciens* (96.0–96.9% sequence similarity). This confirms the previous finding of Rothe et al. (1987) concerning the close relationship between *Blastobacter aggregatus* and *A. tumefaciens* determined by rRNA oligonucleotide catalogue analysis. *Blastobacter denitrificans* on the other hand is most closely related to the type species of the nitrogen-fixing genus *Agromonas* from paddy soil, *Agromonas oligotrophica*, and to *Bradyrhizobium japonicum*. The relationship of *Blastobacter denitrificans* to *Bradyrhizobium japonicum* was first observed by Willems and Collins (1992) based on 16S rRNA sequence similarities and by Green and Gillis (1989) based on rRNA cistron similarities. Willems et al. (2001) showed that the 16S rRNA sequences of photosynthetic

Bradyrhizobium strains, *Agromonas oligotrophica*, and *Blastobacter denitrificans* belong to a well-supported cluster and may represent a separate genus. Further data on the DNA–DNA hybridization levels between the species in this cluster are required to determine the relationships between the members of this phylotype.

Correction of the taxonomic problems within *Blastobacter* is not straightforward because a culture of the type species *Blastobacter henricii* was never isolated. Hugenholtz et al. (1994) proposed that a new type species for the genus be designated. In the absence of physiological and phylogenetic information about the type species, there is no way of knowing to which phylogenetic lineage the true blastobacters as described by Zavarzin (1961) belong. One solution is to reserve the genus *Blastobacter* at this time for *Blastobacter henricii* in case a culture matching the description can be isolated from the same habitat in the future, and to describe new genera for the other species. Given that the description of *B. henricii* is too limited for reliable assignment of isolates to the species, a better solution is to retain the genus for one of the phylotypes of extant species. Circumscription of the

genus to include *B. henricii*, *B. aggregatus*, and *B. capsulatus* would be straightforward. The taxonomic position of *Blastobacter denitrificans*, on the other hand, is more problematic given its close relationship with the budding bacterium *Agromonas oligotrophica* and photosynthetic *Bradyrhizobium* strains. A reasonable taxonomic solution, therefore, will be to retain *Blastobacter aggregatus* and *Blastobacter capsulatus* in the genus *Blastobacter* on the grounds that they belong to a well-supported phylogenetic group sufficiently distant from related taxa at the genus level and that *Blastobacter aggregatus* appears to have characteristics closest to the original description of *Blastobacter* (Hirsch and Müller, 1985). We propose that the species *B. henricii*, *B. aggregatus*, and *B. capsulatus* be retained in the genus *Blastobacter* and that *Blastobacter denitri-*

ficans be transferred to the genus *Agromonas*. DNA–DNA hybridization between *Blastobacter denitrificans* and *Agromonas oligotrophica* will be required to determine species relationships and nomenclatural priority within the genus *Agromonas*. The budding bacterium *Gemmobacter aquatilis* was shown to be most closely related to *Rhodobacter capsulatus* by rRNA catalogue analysis (Rothe et al., 1987). A complete 16S rRNA sequence is required to determine the exact phylogenetic position of this phenotypically and phylogenetically related species. Determination of the phylogenetic positions of the invalid species "*B. aminooxidans*" and "*B. viscosus*" will be required to resolve the remaining taxonomic uncertainty in the genus.

List of species of the genus *Blastobacter*

1. *Blastobacter henricii* Zavarzin 1961, 962^{AL}

hen.ri'ci.i. M.L. gen. n. *henricii* of Henrici; named for A. Henrici, an American microbiologist who may have been the first to see bacteria belonging to the genus *Blastobacter*.

Cells are rod-, wedge-, or club-shaped, $0.7\text{--}1.0 \times 2.0\text{--}4.5\text{ }\mu\text{m}$. Cells form rosettes by attaching to each other with the nonreproductive, frequently tapered pole. A glistening corpuscle was seen in the center of the rosette. Single buds (spherical to oblong) are formed terminally on the blunt cell pole. Released buds are $0.3\text{ }\mu\text{m}$ wide and nonmotile. Originally found in a cylinder containing iron-rich forest brook water from Northern Russia and to which shreds of filter paper had been added. Growth was best in a zone of reduced iron at a pH of 6.2. A pure culture was not obtained.

The mol% G + C of the DNA is: unknown.

Type strain: No culture has been isolated.

2. *Blastobacter aggregatus* Hirsch and Müller 1986, 354^{VP} (Effective publication: Hirsch and Müller 1985, 284.)

ag.gre.ga'tus. L. adj. *aggregatus* joined together, referring to the frequent formation of rosettes.

Cells are $0.6\text{--}0.8 \times 1.5\text{--}2.3\text{ }\mu\text{m}$, ovoid to rod shaped. Multiplication by rod-shaped buds formed at the narrow and unattached cell pole. Bud cells are motile and attach to each other to form rosettes. Colonies colorless to slightly beige or brownish, round with entire edges, dull to shiny. Liquid cultures turbid. Grow well in dilute media containing peptone, yeast extract, and glucose. Vitamins not required. Grow in presence of up to 36 g/l NaCl. Further characteristics are given in Table BXII.α.150. Isolated from surface water of Lake Hötsee (Holstein, Germany).

The mol% G + C of the DNA is: 60 (T_m).

Type strain: Müller 161, ATCC 43293, DSM 1111, IFAM 1003.

GenBank accession number (16S rRNA): X73041.

3. *Blastobacter capsulatus* Hirsch and Müller 1986, 354^{VP} (Effective publication: Hirsch and Müller 1985, 285.)

cap.su.la'tus. L. n. *capsula* a small chest, capsule; M.L. neut. n. *capsulatus* encapsulated.

Cells are $0.7\text{--}0.9 \times 1.5\text{--}2.3\text{ }\mu\text{m}$, rod shaped to short ovoid, often bent and narrowing on one cell pole. Older cells frequently Y shaped. Buds are produced terminally on the narrow cell pole or occasionally laterally. Cells form capsules. Nonmotile. Rosettes not formed. Grow in liquids

as turbidity or pellicle. Exopolymer produced. Nitrate is reduced without gas formation. Growth occurs in the presence of up to 27 g/l NaCl. Grow well in dilute media containing peptone, yeast extract, and glucose. Vitamins not required. Further characteristics are given in Table BXII.α.150. Isolated from a shallow eutrophic pond near Westensee (Kiel, Germany).

The mol% G + C of the DNA is: 59 (T_m).

Type strain: Müller 216, ATCC 43294, DSM 1112, IFAM 1004.

GenBank accession number (16S rRNA): X73042.

4. *Blastobacter denitrificans* Hirsch and Müller 1986, 354^{VP} (Effective publication: Hirsch and Müller 1985, 285.)

de.ni.tri'fi.cans. L. prep. *de* away from; L. n. *nitrum* soda; M.L. n. *nitrum* nitrate; M.L. v. *denitrifico* denitrify; M.L. part. adj. *denitrificans* denitrifying.

Cells are $0.6\text{--}0.8 \times 1.5\text{--}2.3\text{ }\mu\text{m}$, rod shaped with rounded poles. Rod-shaped buds formed subpolarly, motile with 1–3 subpolar flagella. Do not produce capsules. Rosettes not formed. Growth in liquid media is turbid. Colonies glistening, round with entire edges, initially colorless, later beige to brownish in transmitted light. Grow well in dilute media with peptone, yeast extract, and glucose. Grow well with C_1 compounds such as methanol, formate, or formamide. Denitrification occurs with nitrogen gas formed from nitrate anaerobically. Growth occurs in the presence of up to 27 g/l NaCl. Further characteristics are given in Table BXII.α.150. Isolated from surface water of Lake Plussee (Holstein, Germany).

The mol% G + C of the DNA is: 65 (T_m).

Type strain: Müller 222, ATCC 43295, DSM 1113, IFAM 1005.

GenBank accession number (16S rRNA): X66025.

5. "*Blastobacter aminooxidans*" Doronina, Govorukhina and Trotsenko 1983, 552.

a.mi.no.ox'i.dans. M.L. n. *aminum* amine; M.L. v. *oxido* make acid, oxidize; M.L. part. adj. *aminooxidans* oxidizing amines.

Cells are $0.8\text{--}1.0 \times 1.5\text{--}3.0\text{ }\mu\text{m}$, rod shaped, often pleomorphic, forming Y-shaped cells. Cells frequently possess minute tube-like appendages on one pole. Multiplication is by nonmotile, oval buds formed terminally or laterally. Rosettes not formed. Colonies on agar media with trimethylamine or glucose are yellow, 2 mm in diameter, convex, round, glistening, and opaque. Colonies have a smooth surface, an entire edge, uniform consistency, and are vis-

cous. Exopolymer not produced. Vitamins are not required. Gelatin and starch are hydrolyzed; milk is alkalized, but not peptonized or coagulated. Nitrates are reduced to nitrite. Acid but no gas produced from glucose. Methyl red and Voges-Proskauer tests are negative. Carbon sources utilized include monomethylamine, dimethylamine, or trimethylamine, ethanol, butanol, mannitol, xylose, glucose, raffinose, fumarate, malate, and lactate. Nitrogen sources utilized are ammonium, nitrate and peptone, methylated amines, and certain amino acids, but not nitrite. Autotrophic growth occurs in an atmosphere of $H_2/CO_2/O_2$. The main fatty acids of whole cells are $C_{18:1}$ (48%) and $C_{19:0}$ (27%). Further characteristics are given in Table BXII.α.150. Isolated from activated sludge of a sewage purification system at a pulp and paper mill.

The mol% G + C of the DNA is: 69 (T_m).

Deposited strain: 14a (Culture Collection Institute of Microbial Biochemistry and Physiology, Academy of Sciences, Russia.)

6. “**Blastobacter viscosus**” Loginova and Trotsenko 1979, 650. *vis.co* *sus*. L. adj. *viscosus* sticky.

Cells are rod shaped to pleomorphic, often bent, and occasionally branched. Microcapsules may be present. Cell

size range is $0.5\text{--}0.9 \times 1.0\text{--}3.2 \mu\text{m}$. Buds are produced polarly and laterally and are ovoid and nonmotile. Rosettes not formed. Colonies on agar containing peptone and glucose are 3–4 mm in diameter, round, convex, shiny, and opaque, with an even edge, smooth surface, and a slimy consistency. Colony pigmentation is yellow. In liquid medium with methanol and glucose, growth occurs as turbidity and a slimy sediment. Vitamins not required. Exopolysaccharide containing galactose, glucose, rhamnose, xylose, and glucuronic acid is formed. Carbon sources utilized are galactose and sucrose but not methane, alanine, or glycine. Autotrophic growth occurs in an atmosphere of $H_2/O_2/CO_2$ or with methanol. Utilizes ammonium, peptone, and amino acids as nitrogen sources. Gelatin, starch, and cellulose are not hydrolyzed. Acid but no gas produced from glucose. Milk alkalized but not peptonized or coagulated. Acetoin, indole, or H_2S not produced. The main fatty acids of PYG agar-grown cells are $C_{18:1}$ (63%) and $C_{16:0}$ (14%). Isolated from activated sludge of the drainage system of the Baikal paper mill in Russia.

The mol% G + C of the DNA is: 66 (T_m).

Deposited strain: 7d, UCMV-1439D (Culture Collection, Institute Microbial Biochemistry and Physiology, Academy of Sciences, Pushchino, Russia.)

Genus V. **Bosea** Das, Mishra, Tindall, Rainey and Stackebrandt 1996, 985^{VP}

SUBRATA K. DAS

Bos e.a. M.L. gen. n. *Bosea* named after Sir J.C. Bose, the founder of Bose Institute, Calcutta, India.

Rod shaped cells, $0.85 \times 1.4\text{--}1.6 \mu\text{m}$. Gram negative. **Aerobic.** Occurring singly. **Motile by a single polar flagellum.** Optimal temperature for growth: 30–32°C, range; 20–37°C. Optimal pH: 7.5–8.0; range, 6.0–9.0. **Chemolithoheterotrophic**, able to obtain energy from the oxidation of reduced sulfur compounds in the presence of organic carbon. No autotrophic growth occurs. Catalase- and oxidase-positive. Found in cultivated soil.

The mol% G + C of the DNA is: 68.2.

Type species: ***Bosea thiooxidans*** Das, Mishra, Tindall, Rainey and Stackebrandt 1996, 985.

FURTHER DESCRIPTIVE INFORMATION

Colonies on GYM medium¹ supplemented with $Na_2S_2O_3 \cdot 5H_2O$ and an organic substrate (Das et al., 1996) are round, circular, smooth, mucoid, and cream colored. *Bosea thiooxidans* has a single polar flagellum, which is observed with electron microscopy (Fig. BXII.α.178). Pigments are not produced.

The phospholipid composition consists of phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline, diphosphatidyl glycerol, and an amino lipid. The cellular fatty acid composition of *B. thiooxidans* strain BI-42 is presented in Table BXII.α.151.

Neither ammonia nor nitrate serves as the sole nitrogen source for growth. *B. thiooxidans* lacks glutamate synthase activity and therefore is a glutamate auxotroph.

In batch culture, growth and thiosulfate oxidation by this organism in GYM medium without any extra carbon source is gra-

tuitous, since it does not increase the growth yield. The low cell yield indicates that, like other chemolithoheterotrophic organisms such as *Thiobacillus* Q (Gommers and Kuenen, 1988) and *Catenococcus thiocycli* (Sorokin, 1992), *B. thiooxidans* cannot assimilate carbon dioxide autotrophically; a failure to detect ribulose 1,5-bisphosphate carboxylase is consistent with this conclusion. A marked stimulation of growth does occur when GYM medium is supplemented with sodium succinate in addition to thiosulfate. The growth yield varies with the final concentration of thiosul-

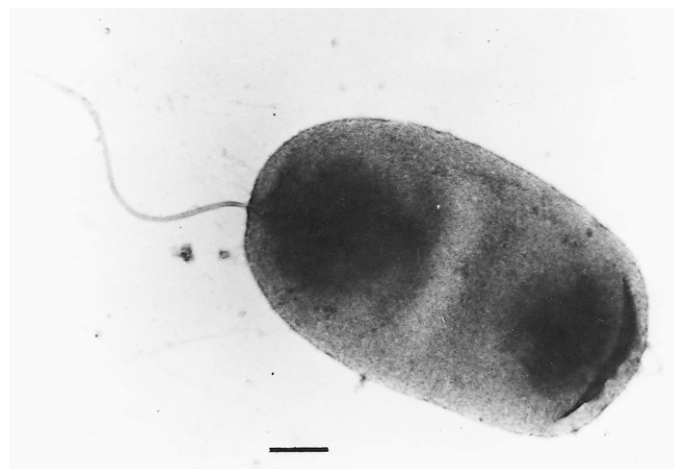


FIGURE BXII.α.178. Transmission electron micrograph of *Bosea thiooxidans* showing the single, polar flagellum. Bar = 200 nm. (Reprinted with permission from S.K. Das et al, International Journal of Systematic Bacteriology 46: 981–987, 1996, ©Society for General Microbiology.)

1. GYM-medium (Glutamate-yeast extract-mineral salts medium), g/l distilled water: Na_2HPO_4 , 4.0; KH_2PO_4 , 1.5; $MgCl_2 \cdot 5H_2O$, 0.1; sodium glutamate, 0.5; and yeast extract powder, 0.1. Final pH, 8.0.

TABLE BXII.α.151. Cellular fatty acid composition of *Bosea thiooxidans* (strain BI-42)

Fatty acids	Designation	% ^a
<i>Nonhydroxylated acids:</i>		
Pentadecanoic acid	C _{15:0}	2.33
<i>cis</i> -Hexadec-9-enoic acid	C _{16:1} ω7c	3.7
Hexadecanoic acid	C _{16:0}	7.58
<i>cis</i> -Hepta-9-enoic acid	C _{17:1} ω8c	3.56
<i>cis</i> -9,10-Methylene hexadecanoic acid	C _{17:0} cyclo	2.08
Heptadecanoic acid	C _{17:0}	4.82
<i>cis</i> -Octadec-9-enoic acid	C _{18:1} ω7c	61.55
Octadecanoic acid	C _{18:0}	0.67
<i>cis</i> -Cyclo-10,11-methylene octadecanoic acid	C _{19:0} cyclo ω8c	8.35
10-Methyl octadecanoic acid	C _{19:0} 10CH ₃	0.71
<i>Hydroxylated acids:</i>		
3-Hydroxypentadecanoic acid	C _{15:0} 3OH	0.49
3-Hydroxyhexadecanoic acid	C _{16:0} 3OH	3.25
3-Hydroxyheptadecanoic acid	C _{17:0} 3OH	0.9

^aPercentages of the total fatty acids. (Adapted by permission of the Society for General Microbiology and with permission from S.K. Das et al., International Journal of Systematic Bacteriology 46: 981–987, 1996.)

fate; maximum growth (A_{660} , 1.2–1.3) occurs at a concentration of 5 g/l. Under these growth conditions there is a stoichiometric conversion of thiosulfate to sulfate with a concurrent decrease in the pH of the medium from 8.0–6.6. The thiosulfate is almost consumed within 36 h and its oxidation enhances heterotrophic carbon assimilation. Cytochrome *c*, *b*, and *aa*₃ are present. Cytochrome *c* occurs in both the soluble and membrane fractions whereas cytochrome *b* is membrane bound only. The genetic regulation of thiosulfate metabolism by *B. thiooxidans* has been studied by transposon insertion mutagenesis (Das and Mishra, 1996). Several thiosulfate metabolism defective mutants were isolated and a comparative enzymatic study with the wild type strain suggested that the enzymes thiosulfate oxidase, sulfite oxidase, and cytochrome *c*_{550–552} are mainly responsible for thiosulfate oxidation.

The MICs (minimum inhibitory concentrations) of several antibiotics for the type strain are (μg/ml): streptomycin, 120; tetracycline, 10; neomycin, 40; chloramphenicol, 20; and rifampicin, 15. Ampicillin fails to inhibit growth even at 200 μg/ml.

ENRICHMENT AND ISOLATION PROCEDURES

For isolation, use cultivated soil from different agricultural fields. Place the soil samples (25 g) in Petri plates and moisten with sterile distilled water. Enrich by adding sodium thiosulfate, sodium sulfite, and S⁰. After thorough mixing, incubate the plates at 30°C for 10 d. Further enrich in mineral salt broth (pH 8.0) supplemented with phenol red (0.02 g/l) and either Na₂S₂O₃·5H₂O (5 g/l) or thiosulfate plus yeast extract (5 g/l). The mineral salts medium contains (per liter): Na₂HPO₄, 4.0 g; KH₂PO₄, 1.5 g; MgCl₂·5H₂O, 0.1 g; NH₄Cl, 1.0 g; and trace metal solution (Vishniac and Santer, 1957), 2 ml. Adjust the pH to 8.0 with 4 N NaOH. Inoculate 1 g of enriched soil from each Petri plate into a 250-ml flask containing 50 ml of enrichment medium and incubate in a water bath shaker at 220 rpm at 30°C. After two days, if a yellow color develops (indicating acid formation),

plate serial dilutions onto mineral salts-thiosulfate agar and mineral salts-thiosulfate-yeast extract agar. After 4 days at 30°C a few colonies surrounded by yellow halos should appear on mineral salts-thiosulfate-yeast extract agar (but not on the mineral salts-thiosulfate agar). Select these colonies individually, streak and restreak onto similar media until a pure culture is obtained.

MAINTENANCE PROCEDURES

For short-term preservation, streak cultures onto mixed substrate agar or Luria agar plates, and incubate until growth becomes visible. Store the plates at 4°C and transfer at two-month intervals. Alternatively, grow cultures to the early stationary growth phase, freeze them in the growth medium with 15% glycerol, and store at –20°C. The cultures should remain viable for several months to a year or more. Lyophilization is the method of choice for long-term preservation.

DIFFERENTIATION OF THE GENUS *BOSEA* FROM OTHER GENERA

Bosea is similar to the genus *Thiobacillus* in regard to mol% G + C content of its DNA, respiratory quinones, and ability to oxidize inorganic sulfur compounds. However, it possesses hydroxy fatty acids in combination with 10-methyl and cyclic fatty acids—a feature that is not found in autotrophic and facultatively chemolithoautotrophic sulfur oxidizers or any other member of the alpha group of the *Proteobacteria*. The presence of ubiquinone (Q-10), phosphatidyl choline and a high content of unsaturated nonhydroxy fatty acid C_{18:1} ω7c is similar to that of *Methylobacterium*; however, the latter is not able to oxidize inorganic sulfur compounds. Unlike *Thiobacillus* sp., *Bosea* grown on mixed substrate agar does not produce sulfur deposits on colony surfaces even though both have a chemolithoheterotrophic mode of thiosulfate oxidation. Although both *Bosea* and *Thiobacillus versutus* denitrify under heterotrophic growth conditions, the absence of denitrification under mixed substrate growth conditions differentiates *Bosea* from *Thiosphaera pantotropha* and *T. versutus*.

TAXONOMIC COMMENTS

16S rDNA sequence analysis of the type strain indicates that *Bosea* constitutes a new genus of the *Alphaproteobacteria*. The 16S rDNA sequence has been deposited in the EMBL database under accession number X81044. A total of 1377 nucleotides of the 16S rDNA of the type strain were amplified and sequenced. The sequence was most similar to that of *Beijerinckia indica* (level of similarity, 92.8%) and to those of *Rhodopseudomonas palustris*, *Nitrobacter winogradskyi*, *Blastobacter denitrificans*, and *Bradyrhizobium japonicum* (levels of similarity 92.0–92.5%) of the *Alphaproteobacteria*. The distance matrix phylogenetic tree based on dissimilarity values of *B. thiooxidans* as compared to those of nineteen reference strains of the *Alphaproteobacteria* indicated a new lineage located between the methylotrophs, the genus *Beijerinckia*, and the *R. palustris* group (Fig. BXII.α.179). No close relationship was found between *B. thiooxidans* and other sulfur oxidizing bacteria such as *Thiobacillus acidophilus* and *Acidiphilium* species (levels of 16S rDNA similarity were less than 88%).

List of species of the genus *Bosea*

1. ***Bosea thiooxidans*** Das, Mishra, Tindall, Rainey and Stackebrandt 1996, 985^{VP}

thi.o.ox'i.dans. Gr. n. *thion* sulfur; M.L. v. *oxido* make acid, oxidize; M.L. part. adj. *thiooxidans* oxidizing sulfur.

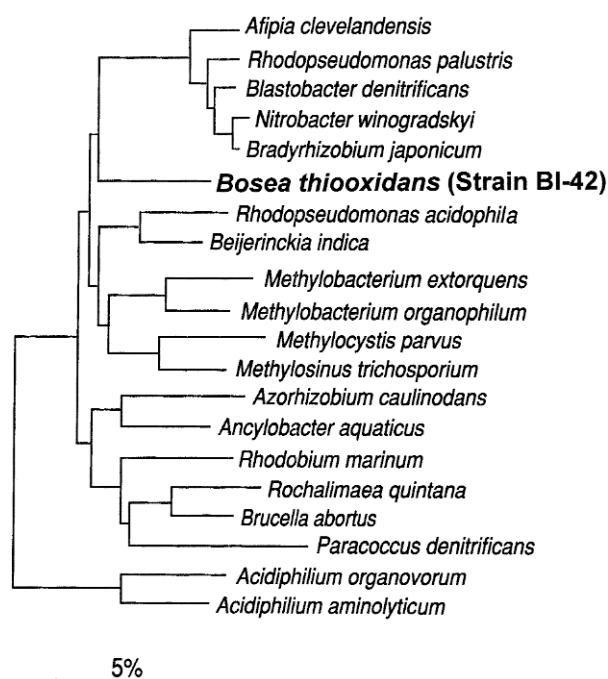


FIGURE BXII.α.179. Phylogenetic tree based on dissimilarity values, showing the relationships between *Borea thiooxidans* (strain BI-42) and related reference organisms. Note that *Rochalimaea quintana* has been transferred to *Bartonella* and is now *Bartonella quintana*. Bar = 5% nucleotide difference. (Reprinted with permission from S.K. Das et al, International Journal of Systematic Bacteriology 46: 981–987, 1996, ©Society for General Microbiology.)

Characteristics are as described for the genus. Glutamate, glutamine, and aspartate, but not ammonium salts, nitrate, or urea, can serve as nitrogen sources. Glutamate auxotrophy occurs and the organisms require yeast extract powder (0.1 g/l) for growth. There is no anaerobic growth in the presence or absence of nitrate; however, under micro-aerobic conditions denitrification occurs with gas production in GYM medium containing malate, succinate, glucose, or sucrose. Gas is not formed after the addition of thio-sulfate to the above growth media.

Organic compounds supporting heterotrophic growth include glucose, fructose, sorbose, xylose, pyruvate, rhamnose, ribose, arabinose, galactose, citrate, gluconate, succinate, malate, acetate, glutamine, proline, aspartic acid, cysteine, serine, asparagine, alanine, and lysine. The following do not support growth: formate, glycerol, mannitol, raffinose, leucine, glycine, isoleucine, methionine, tyrosine, tryptophan, phenylalanine, glyoxylate, lactate, propionate, salicylate, butyrate, cyclohexanol, *p*-amino benzoate, or methanol.

Growth occurs on Simmons citrate agar and MacConkey agar. Tests using methyl red, urease, starch hydrolysis, indole production, H₂S production, Voges–Proskauer, gelatin hydrolysis, and pigment production are negative.

Tetrathionate is oxidized slowly. Sulfite, thiocyanate and S⁰ do not support growth.

Ubiquinone 10 is the major ubiquinone.

Isolated from agricultural field soil near Calcutta, India.

The mol% G + C of the DNA is: 68.2 (*T_m*).

Type strain: BI 42, ATCC 700366, DSM 9653.

GenBank accession number (16S rRNA): X81044.

Genus VI. *Nitrobacter* Winogradsky 1892, 127^{AL} Nom. Cons. Opin. 23 Jud. Comm. 1958, 169

EVA SPIECK AND EBERHARD BOCK

Ni.tro.bac'ter. L. n. *nitrum* nitrate; M.L. n. *bacter* the masc. form of Gr. neut. n. *bactrum* a rod; M.L. masc. n. *Nitrobacter* nitrate rod.

Pleomorphic rod- or pear-shaped cells 0.5–0.9 × 1.0–2.0 μm. Cells reproduce by budding or binary fission. **Intracytoplasmic membranes occur as a polar cap of flattened vesicles** in the cell periphery. Gram negative. Cells may be motile by means of a single polar to subpolar flagellum. **Grows lithoautotrophically and chemoorganotrophically.** Under oxic conditions, nitrite is the preferred energy source and carbon dioxide is the main source of carbon. **Aerobic, but also capable of anaerobic respiration with nitrate.** Under anoxic conditions nitrate is reduced to nitrite, nitric oxide, and nitrous oxide. Occurs in aerobic and micro-aerophilic habitats where organic matter is mineralized.

The mol% G + C of the DNA is: 59–62.

Type species: *Nitrobacter winogradskyi* Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 552 emended mut. char. Watson 1971, 264.

FURTHER DESCRIPTIVE INFORMATION

Nitrobacter cells are facultative lithoautotrophs that obtain energy from the oxidation of nitrite to nitrate during lithoautotrophic growth. The main source of carbon is carbon dioxide. Alternatively, pyruvate, formate and acetate can serve as energy and carbon sources even in the absence of nitrite. The optimal pH

range for growth is 7.5–8.0; the temperature range for growth is 5–37°C with an optimum between 28 and 30°C. Heterotrophic growth is often unbalanced and is accompanied by the formation of large quantities of poly-β-hydroxybutyrate granules. Glycogen and polyphosphates granules are also found as cytoplasmic inclusions. Carboxysomes are present in most but not all species of *Nitrobacter*.

Additional details and a comparison of the biochemical properties of *Nitrobacter* to those of other nitrite-oxidizing genera can be found in the introductory chapter “Lithoautotrophic Nitrite-Oxidizing Bacteria.” Details about the ecology of nitrite-oxidizing bacteria and of the phylogeny of these organisms are described in the introductory chapter, Nitrifying Bacteria.

Nitrobacter cells may be either rod-shaped (Fig. BXII.α.180) or pear-shaped. A pear-shaped cell of *Nitrobacter* with a polar cap of intracytoplasmic membranes is shown in Fig. BXII.α.181. The cell wall differs from that found in other Gram-negative bacteria in that the inner side of the wall is more electron dense than the outer one. A similar asymmetry occurs in the cytoplasmic and intracytoplasmic membranes (Fig. BXII.α.181); carboxysomes are formed (Fig. BXII.α.181). Freeze-etched or negatively stained preparations reveal that the inner surface of these mem-

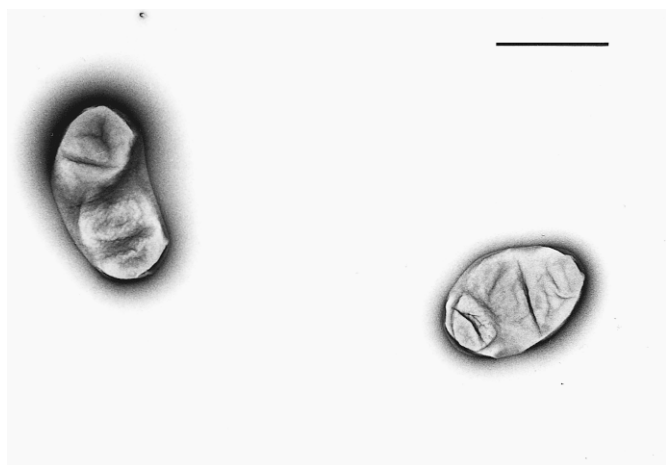


FIGURE BXII.α.180. Negatively stained short rods of *Nitrobacter winogradskyi* Engel. Bar = 1 μm.

branes is covered with densely packed particles with a size of 8–10 nm (Tsien et al., 1968, Remsen and Watson, 1972, Sundermeyer and Bock, 1981, Sundermeyer-Klinger et al., 1984). These particles are composed of the nitrite oxidoreductase enzyme (NOR). Electron microscopy of the isolated enzyme reveals uniform particles with a size of 8 nm (Meincke et al., 1992). Particulate membranes have been labeled with monoclonal antibodies recognizing the α - and β -subunits of the NOR (Spieck et al., 1996a). The particle locations correlate with immunogold-labeling of the α - and β -subunits of nitrite oxidoreductase; labeling of the α -subunit in *Nitrobacter hamburgensis* is shown in Fig. BXII.α.182. The enzyme forms a periodic arrangement in paired rows (Fig. BXII.α.183); a digital image processing analysis of this two-dimensional structure is shown in Fig. BXII.α.184. The molecular weight of a single particle is 186 kDa, suggesting that it is most likely composed of an $\alpha\beta$ -heterodimer (Spieck et al., 1996b); the α -subunit has a mass of 115–130 kDa and the β -subunit a mass of 65 kDa.

Lithotrophic growth is slow. The generation time varies from 8 h to several days.

In *Nitrobacter* the concentration of NOR varies with growth conditions. Synthesis of the enzyme is induced by nitrite or nitrate, and the enzyme is the major constituent of nitrite-oxidizing membranes (Bock et al., 1991). In *Nitrobacter hamburgensis* the nitrite-oxidizing activity of mixotrophically grown cells is higher than that of autotrophically grown cells (Milde and Bock, 1985). During heterotrophic growth the NOR is repressed by a factor of more than 90%. When O_2 is absent, NOR shows nitrate reductase activity (Sundermeyer-Klinger et al., 1984). Furthermore, a membrane-bound nitrite reductase which transformed nitrite to NO in low oxygen conditions has been copurified with the NOR (Ahlers et al., 1990). Depending upon the isolation procedure, the NOR is obtained as complexes containing 2–3 subunits (Tanaka et al., 1983; Sundermeyer-Klinger et al., 1984). The membrane-associated α -subunit (115–130 kDa) and β -subunit (65 kDa) can be solubilized by heat treatment at 55°C. Investigations by electron paramagnetic resonance spectroscopy (EPR) showed that the catalytically active enzyme includes molybdenum and iron-sulfur centers which are involved in the transformation of nitrite to nitrate (Meincke et al., 1992). The cytochromes a_1 and c_1 have been coisolated with NOR in procedures

employing different detergents. Additional major proteins of the intracellular membranes had molecular masses of 14, 28, and 32 kDa. The 32-kDa protein is the γ -subunit of the NOR (Sundermeyer-Klinger et al., 1984). Tanaka et al. (1983) suggested that the subunit structure of the NOR (cytochrome a_1c_1) of *Nitrobacter winogradskyi* (254 kDa) consists of proteins with molecular masses of 55, 29 and 19 kDa. The pH optimum of the isolated enzyme was 8.0, and the K_m value for nitrite was determined to be 0.5–2.6 mM by Tanaka et al. (1983) and 3.6 mM by Sundermeyer-Klinger et al. (1984). A K_m value of about 0.9 mM was calculated for the reduction of nitrate. Kirstein and Bock (1993) identified the genes of the NOR and obtained the amino acid sequence of the NOR β -subunit of *Nitrobacter hamburgensis* X14. This protein contained four cysteine clusters (probably three [4Fe-4S] and one [3Fe-4S]) and shared significant sequence similarities with the β -subunits of the dissimilatory nitrate reductases (NRA, NRZ) of *Escherichia coli*. The β -NOR is thought to function as an electron-channeling protein between the nitrite-oxidizing α -subunit and the membrane-integrated electron transport chain. Recently, the genes encoding the α -subunit of the NOR were sequenced in the four described species of *Nitrobacter* (Degrange, personal communication). As in the case of the β -NOR, the *Nitrobacter* α -NORs possess significant sequence similarities to the α -subunits of several dissimilatory nitrate reductases (e.g., *Escherichia coli* or *Pseudomonas* species).

ENRICHMENT AND ISOLATION PROCEDURES

Nitrite oxidizers can be isolated using a mineral medium containing nitrite; the compositions of media for lithotrophic, mixotrophic, and heterotrophic growth are given in Table BXII.α.152. Serial dilutions of enrichment cultures must be incubated for one to several months in the dark. Since nitrite oxidizers are sensitive to high partial pressures of oxygen, cell growth on agar surfaces is limited. Pure cultures of *Nitrobacter alkalicus* were obtained by multiple passages in liquid medium of colonies from nitrite agar (Sorokin et al., 1998). Nitrite oxidizers can be separated from heterotrophic contaminants by Percoll gradient centrifugation and subsequent serial dilution (Ehrich et al., 1995).

MAINTENANCE PROCEDURES

Nitrifying organisms can survive starvation for more than one year when kept at 17°C in liquid medium. Nevertheless, cells should be transferred to fresh media every four months. Table BXII.α.152 lists three different growth media for nitrite oxidizers. Freezing in liquid nitrogen is a suitable technique for maintenance of stock cultures suspended in a cryoprotective buffer containing sucrose and histidine. When freeze-dried on lavalite or polyurethane, about 0.5% of *Nitrobacter* cells survive for one year (L. Lin, personal communication). Another possibility for the storage of *Nitrobacter* for several years is cultivation in 1l-bottles filled to the top with complex medium and closed by a screw top. Glycerol should be used instead of pyruvate to keep the pH-value stable for a long period. Since the bacteria are able to oxidize nitrite to nitrate aerobically and subsequently able to reduce the nitrate anaerobically, a high cell yield can be obtained using this method (Freitag et al., 1987).

DIFFERENTIATION OF THE GENUS *NITROBACTER* FROM OTHER GENERA

The nitrite oxidizers are a diverse group of long or short rods, cocci, and spirilla. The genera *Nitrobacter* and *Nitrococcus* possess

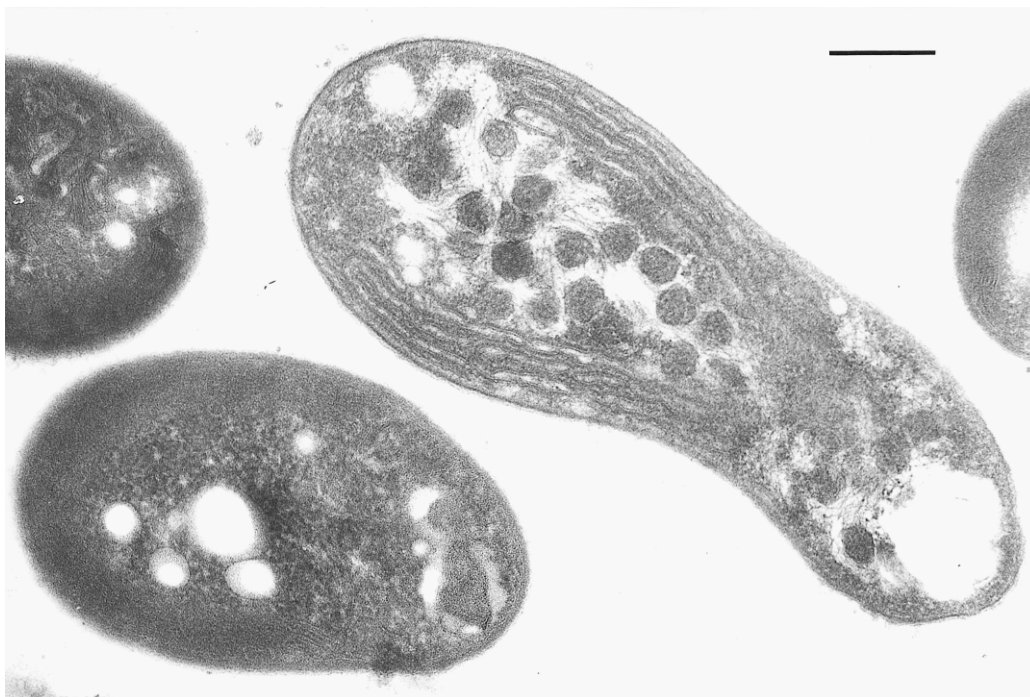


FIGURE BXII.α.181. Ultrathin section of *Nitrobacter vulgaris* strain nevada with intracytoplasmic membranes and carboxysomes. Bar = 250 nm.

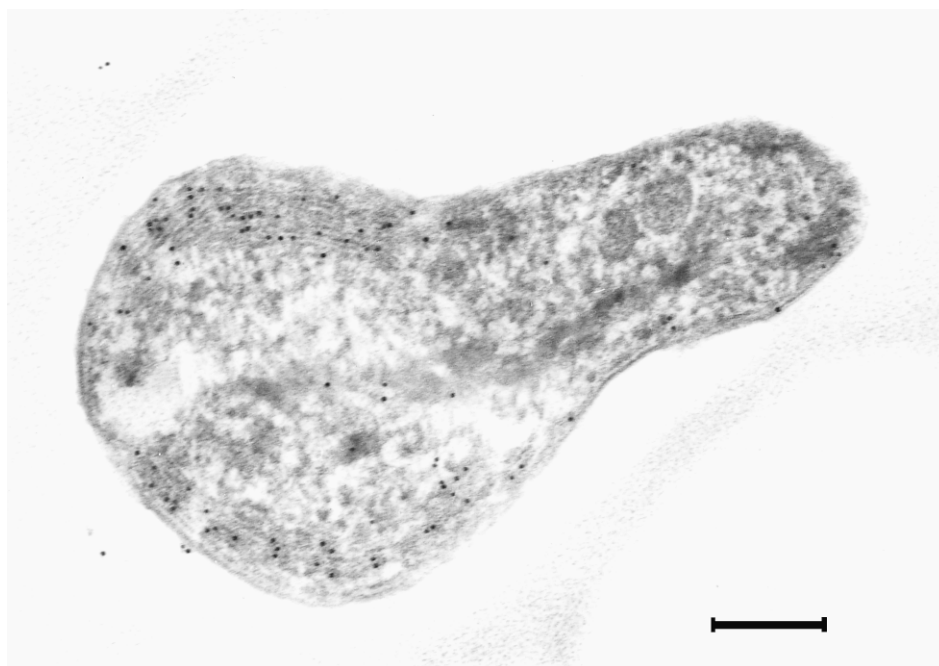


FIGURE BXII.α.182. Ultrathin section of *Nitrobacter hamburgensis* $\times 14$. The nitrite oxidoreductase (NOR) was localized by immunogold-labeling at the cytoplasmic and intracytoplasmic membranes with monoclonal antibodies (MAbs 153-2) recognizing the α -subunit. (Reproduced with permission from E. Spieck et al., FEMS Microbiology Letters 139: 71–76, 1996, ©Elsevier Science B.V., Amsterdam.) Bar = 250 nm.

a complex arrangement of intracytoplasmic membranes in the form of flattened vesicles or tubes. The taxonomic categorization is based on the work of Sergei and Helene Winogradsky (Winogradsky, 1892). Traditionally, the classification of genera is per-

formed primarily on cell shape and arrangement of intracytoplasmic membranes. So far, four morphologically distinct genera (*Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*) have been described; the four genera contain a total of eight species (Watson

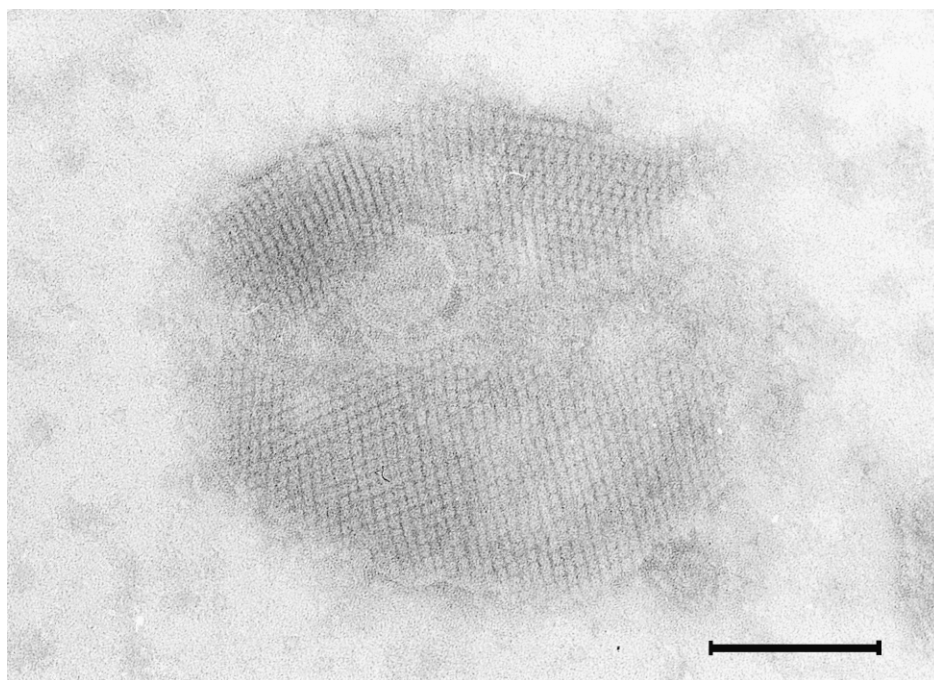


FIGURE BXII.α.183. Isolated negatively stained nitrite-oxidizing membrane of *Nitrobacter hamburgensis* $\times 14$ with crystalline arrays of NOR particles. Bar = 100 nm..(Reproduced with permission from E. Spieck et al., *Journal of Structural Biology* 117: 117–123, 1996, Academic Press Inc., Orlando.)

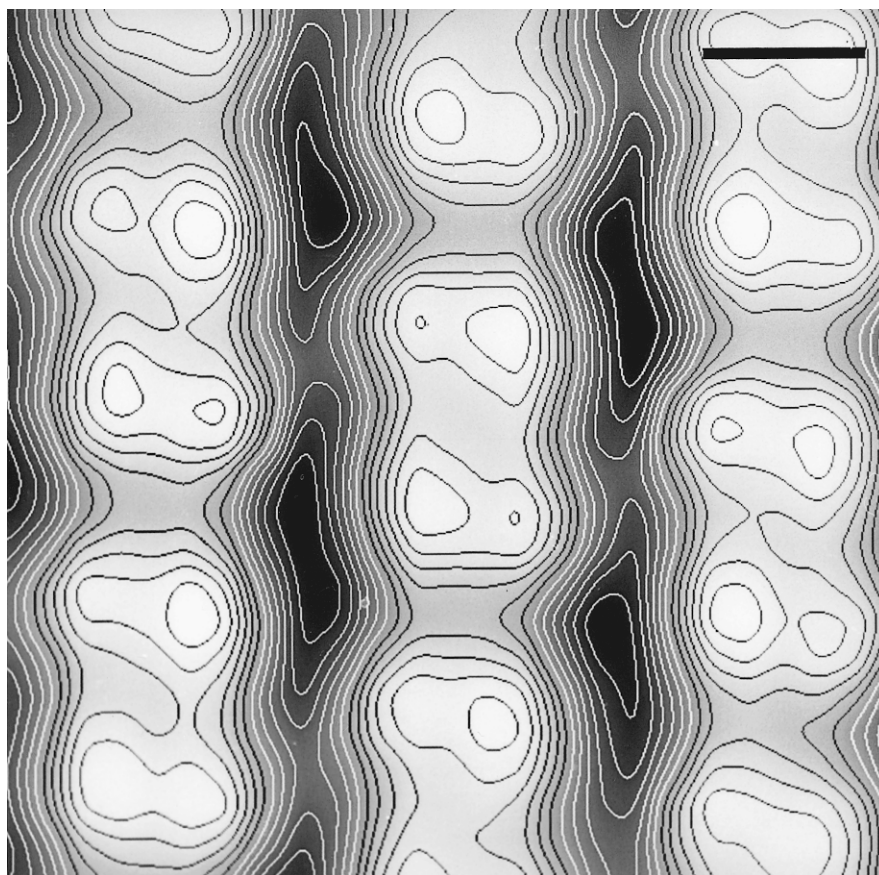


FIGURE BXII.α.184. Image reconstruction of the NOR of *Nitrobacter hamburgensis* $\times 14$ by correlation averaging. The two-dimensional lattice is composed of particle-dimers, arranged in rows. Protein appears bright. Bar = 5nm. (Reproduced with permission from E. Spieck et al., *Journal of Structural Biology* 117: 117–123, 1996, Academic Press Inc., Orlando.)

et al., 1989; Bock and Koops, 1992; Ehrich et al., 1995; Sorokin et al., 1998). Three new species belonging to the genera *Nitrobacter*, *Nitrospina*, and *Nitrospira* are known but remain to be described in the literature. Differential traits of the four genera of nitrite-oxidizing bacteria are given in Table BXII.α.153, further properties in Table BXII.α.154, and fatty acid profiles in Table BXII.α.155. Motility and carboxysomes have been observed in *Nitrobacter* and *Nitrococcus* (Tables BXII.α.153 and BXII.α.154).

Nitrobacter can be separated from the other genera by means of monoclonal antibodies that recognize the key enzyme (Table BXII.α.153). Aamand et al. (1996) developed three monoclonal antibodies (MAbs) that recognize the α- and the β-subunit of the NOR of *Nitrobacter*. The key enzyme NOR is ubiquitous in *Nitrobacter* species; homologous reactions of the MAbs with the NOR of *Nitrobacter winogradskyi* and the NORs of *Nitrobacter vulgaris*, *Nitrobacter hamburgensis*, and *Nitrobacter alkalicus* were demonstrated. *Nitrobacter* sp. BS 5/6, identified as a new species by DNA-

DNA hybridization (Koops, personal communication), was not recognized by the otherwise genus-specific MAb Hyb 153-2. This antibody targets the α-NOR of *Nitrobacter*, whereas MAb Hyb 153-1 recognizes the β-NOSs of both *Nitrobacter* and *Nitrococcus*. The MAb Hyb 153-3 recognizes the β-NOSs of *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira* (Bartosch et al., 1999). Thus, the suite of MAbs permits detection of all known nitrite oxidizers and allows discrimination of *Nitrobacter* and *Nitrococcus* from *Nitrospina* and *Nitrospira*.

TAXONOMIC COMMENTS

16S rDNA sequence analysis showed that all characterized strains of *Nitrobacter* form a closely related assemblage within the *Alphaproteobacteria* (Woese et al., 1984b) and that *Nitrobacter* strains cluster with species of the genera *Rhodopseudomonas*, *Bradyrhizobium*, *Blastobacter* and *Afipia* (Teske et al., 1994) in the family *Bradyrhizobiaceae*. (See Fig. 1 [p. 138] of the introductory chapter "Ni-

TABLE BXII.α.152. Three different media for lithoautotrophic (medium A for terrestrial strains; medium B for marine strains), mixotrophic (medium C), and heterotrophic (medium C without NaNO₂) growth of nitrite oxidizers

Ingredient	Culture medium		
	A ^a	B ^b	C ^{c, d}
Distilled water (ml)	1000	300	1000
Seawater (ml)		700	
NaNO ₂ (mg)	200–2000	69	200–2000
MgSO ₄ ·7H ₂ O (mg)	50	100	50
CaCl ₂ ·2H ₂ O (mg)		6	
CaCO ₃ (mg)	3		3
KH ₂ PO ₄ (mg)	150	1.7	150
FeSO ₄ ·7H ₂ O (mg)	0.15		0.15
Chelated iron (13%, Geigy) (mg)		1	
Na ₂ MoO ₄ ·2H ₂ O (μg)		30	
(NH ₄) ₂ Mo ₇ O ₂₄ ·4H ₂ O (μg)	50		50
MnCl ₂ ·6H ₂ O (μg)		66	
CoCl ₂ ·6H ₂ O (μg)		0.6	
CuSO ₄ ·5H ₂ O (μg)		6	
ZnSO ₄ ·7H ₂ O (μg)		30	
NaCl (mg)	500		500
Sodium pyruvate (mg)			550
Yeast extract (Difco) (mg)			1,500
Peptone (Difco) (mg)			1,500
pH adjusted to ^e	8.6	6	7.4

^aFor terrestrial strains from Bock et al. (1983).

^bFor marine strains modified from Watson and Waterbury (1971).

^cFor terrestrial strains from Bock et al. (1983).

^dFor heterotrophic growth medium C without NaNO₂ is used.

^eAfter sterilization pH should be 7.4–7.8.

TABLE BXII.α.153. Differentiation of the four genera of nitrite-oxidizing bacteria

Characteristic	<i>Nitrobacter</i>	<i>Nitrococcus</i>	<i>Nitrospina</i>	<i>Nitrospira</i>
Phylogenetic position	<i>Alphaproteobacteria</i>	<i>Gamma</i> <i>proteobacteria</i>	<i>Deltaproteobacteria</i> (preliminary)	Phylum <i>Nitrospira</i>
Morphology	Pleomorphic short rods	Coccoid cells	Straight rods	Curved rods to spirals
Intracytoplasmic membranes	Polar cap	Tubular	Lacking	Lacking
Size (μm)	0.5–0.9 × 1.0–2.0	1.5–1.8	0.3–0.5 × 1.7–6.6	0.2–0.4 × 0.9–2.2
Motility	+	+	–	–
Reproduction:	Budding or binary fission	Binary fission	Binary fission	Binary fission
Main cytochrome types ^a	<i>a</i> , <i>c</i>	<i>a</i> , <i>c</i>	<i>c</i>	<i>b</i> , <i>c</i>
Location of the nitrite oxidizing system on membranes	Cytoplasmic	Cytoplasmic	Periplasmic	Periplasmic
MAB-labeled subunits (kDa) ^b	130 and 65	65	48	46
Crystalline structure of membrane-bound particles	Rows of particle dimers	Particles in rows	Hexagonal pattern	Hexagonal pattern

^aLithoautotrophic growth.

^bMAbs, monoclonal antibodies.

TABLE BXII.α.154. Properties of the nitrite-oxidizing bacteria

Characteristic	<i>Nitrobacter winogradskyi</i>	<i>Nitrobacter alkalicus</i>	<i>Nitrobacter hamburgensis</i>	<i>Nitrobacter vulgaris</i>	<i>Nitrococcus mobilis</i>	<i>Nitrospina gracilis</i>	<i>Nitrospira marina</i>	<i>Nitrospira moscoviensis</i>
Mol% G + C of the DNA	61.7	62	61.6	59.4	61.2	57.7	50	56.9
Carboxysomes	+	—	+	+	+	—	—	—
Habitat:								
Fresh water	+			+				
Waste water	+			+				
Brackish water				+				
Oceans	+				+	+	+	
Soda lakes		+						
Soil	+		+	+				
Soda soil		+						
Stones	+			+				
Heating systems								+

TABLE BXII.α.155. Primary fatty acids of the described species of nitrite-oxidizing bacteria^{a,b}

Fatty acid	<i>Nitrobacter winogradskyi</i> Engel	<i>Nitrobacter alkalicus</i> AN4	<i>Nitrobacter hamburgensis</i> X14	<i>Nitrobacter vulgaris</i> Z	<i>Nitrococcus mobilis</i> 231	<i>Nitrospina gracilis</i> 3	<i>Nitrospira marina</i> 295	<i>Nitrospira moscoviensis</i> M1
C _{14:1} ω5c						+		
C _{14:0}	+		+		+	+++	+	+
C _{16:1} ω9c							+++	++
C _{16:1} ω7c	+	+	+	+	+++	+++		
C _{16:1} ω5c							+++	+++
C _{16:0} 3OH						+		+
C _{16:0}	++	++	++	++	+++	++	+++	+++
C _{16:0} 11CH ₃							+	+++
C _{18:1} ω9c	+		+			+	+	
C _{18:1} ω7c	+++	++++	++++	++++	+++	+	+	+
C _{18:0}	+	+	+		+	+	++	+
C _{19:0} cyclo ω7c	+	+	+	+	+			

^aSymbols: +, <5%; ++, 6–15%; +++, 16–60%; +++, >60%.

^bStirred cultures were grown autotrophically at 28°C (*Nitrospira moscoviensis* at 37°C) and collected at the end of exponential growth. Modified from Lipski et al., (2001).

trifying Bacteria”, Volume 2, Part A.) Common traits of *Nitrobacter*, *Rhodopseudomonas*, and *Blastobacter* include the ability to carry out denitrification and cell division by budding. The oligonucleotide data of Seewaldt et al. (1982) demonstrate a close relationship between the 16S rRNAs of *Nitrobacter winogradskyi*, *Nitrobacter hamburgensis* strain X14, and *Rhodopseudomonas palustris*. *Nitrobacter hamburgensis* strain X14 contains an unusual lipid A with 2,3 diamino-2,3-dideoxyglucose similar to that of *Rhodopseudomonas palustris*; this is significant because lipid A structure is considered to be conserved phylogenetically (Mayer et al., 1983). Furthermore, the soluble cytochrome *c* of *Nitrobacter* resembles the cytochrome *c*₂ of *Rhodopseudomonas viridis* (Yamanaka and Fukumori, 1988). The fact that both *Nitrobacter* spp. and *Rhodopseudomonas palustris* possess complex internal membrane systems suggests a common evolutionary origin. Similar membranes are also present in *Methylobacterium*, the closest methylo-trophic relative of *Nitrobacter*.

ACKNOWLEDGMENTS

We thank Wolfgang Ludwig (Technical University in Munich, Germany) for phylogenetic trees and Dmitry Sorokin (Institute of Microbiology in Moscow, Russia) for providing cultures of *Nitrobacter alkalicus*.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *NITROBACTER*

Cells of the genus *Nitrobacter* have a comparable shape, size and ultrastructure. Since most strains are phenotypically similar, species differentiation is based on the mol% G + C content of the DNA, DNA–DNA hybridization, serological properties, patterns of membrane-bound heme proteins, and slight differences in

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growth characteristics. Differential characteristics are given in Table BXII.α.154 and fatty acid profiles in Table BXII.α.155. *Nitrobacter hamburgensis* and *Nitrobacter vulgaris* differ from *N. winogradskyi* in that mixotrophic growth of *N. hamburgensis* and *N. vulgaris* is more rapid than lithoautotrophic growth; *Nitrobacter*

alkalicus can be separated from the other species by its ability to oxidize nitrite under alkaline conditions.

The DNA–DNA hybridization values between the pairs of species range from 20–49% (Bock et al., 1990). The mol% G + C of the DNA of *Nitrobacter* species ranges from 59–62%. Navarro et al. (1992) investigated the genomic relationships within this genus by examination of rRNA gene restriction patterns and by DNA–DNA hybridization. These authors differentiated three genomic species, two of which corresponded to the described species *N. winogradskyi* and *N. hamburgensis*. (No strains of *N. vulgaris* were examined in that study.) High-resolution phylogenetic investigations of *Nitrobacter* have also been carried out using 16S–23S rRNA intergenic spacer region sequences and partial sequences of the 23S rRNA gene (Grundmann et al., 2000). Anal-

ysis of 16S rDNA sequences showed that species of *Nitrobacter* form a coherent cluster with sequence similarities of 99.2–99.6% (Orso et al., 1994). Seewaldt et al. (1982) obtained an S_{AB} value of 0.82 for *N. winogradskyi* and *N. hamburgensis* X14. The 16S rRNA similarity between the newly described species *Nitrobacter alkalicus* and both *N. winogradskyi* and *N. hamburgensis* was 98.6–99.9% (Sorokin et al., 1998). The closest relative of the alkaliphilic species is *N. winogradskyi*. DNA–DNA hybridization studies of strains of *N. vulgaris* indicated that this species might contain subspecies. The hybridization values obtained in this study ranged between 55–97%; the distribution of hybridization values among strains agreed with the distribution of variable physiological traits; both sorts of variation may reflect adaptation to special environments (Bock et al., 1990).

List of species of the genus *Nitrobacter*

1. ***Nitrobacter winogradskyi*** Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 552^{AL} emended mut. char. Watson 1971b, 264.

wi.no.grad'skyi. M.L. gen. n. *winogradskyi* of Winogradsky; named after Winogradsky, the microbiologist who first isolated these bacteria.

Short rods, often pear-shaped, sometimes coccoid with a size of $0.6\text{--}0.8 \times 1.0\text{--}2.0 \mu\text{m}$. Carboxysomes occur as cytoplasmic inclusions. Cells may be motile by a single subpolar to lateral flagellum. Many strains are facultative lithoautotrophs. During mixotrophic growth, nitrite is oxidized first, followed by the oxidation of organic material. Cells can also grow heterotrophically with pyruvate, acetate, and glycerol as energy sources and with yeast extract, casamino acids, peptone, ammonia, and nitrate as nitrogen sources. Under lithoautotrophic and mixotrophic conditions, the generation time varies from 8–14 h and under heterotrophic conditions from 70–100 h. The organism is able to grow anaerobically with nitrate as the electron acceptor (Bock et al., 1988) and forms nitrite, nitric oxide (NO), and nitrous oxide (N₂O). The cells are slightly sensitive to oxygen when undergoing a shift from anoxic to oxic conditions. Nitric oxide may be an alternative substrate for the aerobic oxidation to nitrate. The type strain was isolated from soils. Other habitats are fresh water, oceans, sewage disposal systems and compost piles.

The mol% G + C of the DNA is: 61.7 (Bd).

Type strain: ATCC 25391.

2. ***Nitrobacter alkalicus*** Sorokin, Muyzer, Brinkhoff, Kuenen and Jetten 1999, 1325^{VP} (Effective publication: Sorokin, Muyzer, Brinkhoff, Kuenen and Jetten 1998, 352.) *al.ka'li.cus*. M.L. adj. *alkalicus* alkaline.

Pear-shaped cells $0.6\text{--}0.9 \times 1.2\text{--}1.8 \mu\text{m}$. Cell wall contains an additional external layer with a regular subunit arrangement. In contrast to the other species of this genus, no carboxysomes have been detected. Lithoautotrophic growth with nitrite occurs in a broad pH range from 6.5–10.2 with an optimum at 9.5. Heterotrophic growth not observed. Obligately dependent on the presence of carbonate ions. Strains were isolated from the sediments of soda lakes and from soda soil in Siberia and Kenya. The 16S rRNA gene sequences of strains AN 1, AN 2, and AN 4 are deposited in the GenBank database.

The mol% G + C of the DNA is: 62.0 (T_m).

Type strain: AN 1, LMD 97.163.

GenBank accession number (16S rRNA): AF 069956.

The type strain is stored in the culture collection of the Department of Microbiology, University of Technology, Delft, The Netherlands.

3. ***Nitrobacter hamburgensis*** Bock, Sundermeyer-Klinger and Stackebrandt 2001, 1^{VP} (Effective publication: Bock, Sundermeyer-Klinger and Stackebrandt 1983, 283.)

ham'burgen.sis. M.L. adj. *hamburgensis* pertaining to the city of Hamburg, Federal Republic of Germany, where the organism was first isolated.

Morphology and ultrastructure are as described for the genus. Carboxysomes are present. Motile by a subpolar to lateral flagellum. Grows poorly lithoautotrophically but well mixotrophically. Optimal growth rates are obtained in a mixotrophic medium containing nitrite, pyruvate, yeast extract and peptone. One strain could grow only in the presence of organic material. Both nitrite and organic compounds are metabolized during mixotrophic growth. Growth by dissimilatory nitrate reduction is possible, but the cells are sensitive to oxygen when changing from anoxic to oxic conditions. Isolated from soils in Hamburg (Germany), Yucatan (Mexico) and Corse (France).

The mol% G + C of the DNA is: 61.6 (T_m).

Type strain: X 14.

GenBank accession number (16S rRNA): L11663.

The type strain is stored in the culture collection of the Institute of General Botany, Department of Microbiology, University of Hamburg, FRG.

4. ***Nitrobacter vulgaris*** Bock, Koops, Möller and Rudert 2001, 1^{VP} (Effective publication: Bock, Koops, Möller and Rudert 1990, 109.)

vul.ga'ris. L. adj. *vulgaris* common.

The morphology and ultrastructure are as described for the genus. Carboxysomes are present. Most strains show diphasic growth, oxidizing nitrite first and then organic material. Lithoautotrophic growth is often slower than chemorganotrophic growth. Cells of strain 'Z' double in number in 140 h during lithoautotrophic growth and in 25 h during mixotrophic growth. Either nitrate or oxygen can serve as electron acceptor during heterotrophic growth with acetate or pyruvate as electron donor. Cells produce extracellular polymers as biofilm and form microcolonies. In contrast to the other strains of this genus, cells of *N.*

vulgaris are insensitive to oxygen stress. Only one membrane-bound *c*-type cytochrome of 32 kDa is present, in contrast to *N. winogradskyi* and *N. hamburgensis*, which also possess a 30 kDa *c*-type cytochrome. Strains have been isolated from soils, groundwater, fresh and brackish water, sewage and a termite heap. *N. vulgaris* is the most abundant

species of *Nitrobacter* in concrete and natural building stone.

The mol% G + C of the DNA is: 59.4 (T_m).

Type strain: Z.

Type strain is stored in the culture collection of the Institute of General Botany, Department of Microbiology, University of Hamburg, FRG.

Genus VII. *Oligotropha* Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 391)

ORTWIN O. MEYER

O.li.go.tro'pha. Gr. n. *oligos* little, scanty; *tropha* nourishing, living on few substrates; M.L. fem. n. *Oligotropha* utilizer of few substrates.

Rod-shaped cells, slightly curved, $0.4\text{--}0.7 \times 1.0\text{--}3.0 \mu\text{m}$. **Motility variable**; when present, it is by means of **one subpolar flagellum** (Meyer and Schlegel, 1978). Star-shaped aggregates (rosettes)—held together by polar pili and excreted slime—are formed to a certain extent, especially in the post exponential and stationary growth phases. Gram negative. Colonies are white or cream colored, and no carotenoid pigment is produced. Colony formation on agar plates is slow (one to four weeks, depending on the medium). Aerobic, having a respiratory type of metabolism, with oxygen as the terminal electron acceptor; however, some strains can denitrify. **Facultatively chemolithoautotrophic**. Chemoorganoheterotrophic substrates are usually restricted to the salts of some organic acids such as pyruvate, although some strains can also utilize sugars and amino acids. Dinitrogen is not fixed. Not phototrophic. Not methanotrophic. Isolated from wastewater of sewage treatment settling ponds and from soil.

The mol% G + C of the DNA is: 62.5–63.1.

Type species: *Oligotropha carboxidovorans* (Meyer and Schlegel 1978) Meyer, Stackebrandt and Auling 1994, 182 (Effective publication: Meyer, Stackebrandt and Auling 1993, 391) (*Pseudomonas carboxydovorans* Meyer and Schlegel 1978, 42; *Hydrogenomonas carboxydovorans* Kistner 1954, 186.)

FURTHER DESCRIPTIVE INFORMATION

Cells of *Oligotropha* lack intracytoplasmic membranes or chlorosomes, and thus electron micrographs of the cytoplasm are in accord with the absence of phototrophic or methanotrophic metabolic capabilities (Meyer and Rohde, 1984; Rohde et al., 1984). For electron micrographs of the flagella, see Meyer and Schlegel (1978), Meyer and Rohde (1984), and Rohde et al. (1984). The flagellar fine structure (Aragno et al., 1977) and the cell wall (Walther-Mauruschat et al., 1977) are of type I.

Under heterotrophic conditions (e.g., on the mineral medium of Meyer and Schlegel (1983) supplemented with 0.1% nutrient broth and 0.1% pyruvate), colonies first become visible after about 1 week of incubation. Under chemolithoautotrophic conditions (e.g., the mineral medium of Meyer and Schlegel (1983) in a gas atmosphere of 50% CO, 5% CO₂ and 45% air, or of 50% H₂, 10% CO₂ and 40% air), colonies first become visible after a month or more of incubation. Colony sizes may vary, depending on whether a colony has been developed from a single bacterium or a number of aggregated cells.

Submerged growth in shaken liquid cultures is usually much faster than on solid media. Growth of strain OM5 with CO in submerged culture (e.g., in 30-l fermentors at 30°C) in the mineral medium of Meyer and Schlegel (1983) supplied with the indicated trace elements TS2 and a gas mixture of 20% CO and

80% air at a flow rate of 2 l/min occurs at generation times of 20 h. Under these conditions, exponential growth proceeds to an OD₄₃₆ of 7 and a yield of 1 g wet weight/l. Cultivation of the bacteria with H₂ requires essentially the same conditions as with CO, except that a gas mixture of 5% CO₂, 20% H₂, and 75% air is employed. Under these conditions, the generation time is 15 h, and exponential growth proceeds to an OD₄₃₆ of 7 and a yield of 1 g wet weight/l. Because *O. carboxidovorans* OM5 employs a membrane-bound heterodimeric NiFe-hydrogenase for the chemolithoautotrophic utilization of H₂ (Santiago and Meyer, 1997), Ni must be supplied for chemolithoautotrophic growth with H₂.

The metabolism is generally aerobic and facultatively chemolithoautotrophic (Meyer and Schlegel, 1983). The respiratory metabolism is in accord with the presence of cytochromes (Cypionka and Meyer, 1983b). One strain can reduce nitrate to nitrite (Frunzke and Meyer, 1990). Obligate chemolithoautotrophic strains are not known. So far, only mesophilic strains have been characterized. Chemoorganoheterotrophic substrates utilized under aerobic conditions include the salts of pyruvate, formate, glyoxylate, lactate, ascorbate, acetate, malate, fumarate, oxoglutarate, and oxalate (Meyer et al., 1993). Vitamins are not required. Sugars and amino acids are not used by the strains OM3, OM4, and OM5 of *O. carboxidovorans*, whereas strain OM2 utilizes most sugars and amino acids. Suitable nitrogen sources of all strains are urea, ammonia, nitrate, and nitrite. Nitrate is not assimilated by strain OM5 when CO is the substrate.

The aerobic chemolithoautotrophic utilization of CO by *O. carboxidovorans* OM5 follows this equation: O₂ + 2.19 CO → 1.83 CO₂ + 0.36 cell carbon (Meyer and Schlegel, 1978, 1983). The bacteria oxidize CO for the generation of energy (5 CO + 2.5 O₂ → 5 CO₂) and of reducing equivalents (2 CO + H₂O → 2 CO₂ + 2 × 2 [H]). CO₂ derives from the oxidation of CO and is assimilated in the Calvin-Benson-Bassham-cycle (CO₂ + 2 × 2 [H] → [CH₂O] + H₂O). The sum of these equations (7 CO + 2.5 O₂ + H₂O → 6 CO₂ + [CH₂O]) is in accordance with the actually observed stoichiometry of CO oxidation and indicates that 86% of the CO is oxidized for energy generation and 14% of the CO carbon is assimilated.

Carbon monoxide oxidation in *O. carboxidovorans* OM5 is catalyzed by the heterohexameric metalloenzyme CO dehydrogenase (CO + H₂O → CO₂ + 2 H⁺ + 2 e⁻) that is associated with the inner aspect of the cytoplasmic membrane (Meyer and Rohde, 1984; Rohde et al., 1984; Meyer et al., 2000). CO dehydrogenases are very much conserved in all aerobic CO oxidizing bacteria (Schübel et al., 1995; Santiago et al., 1999), and the CO dehydrogenases from the strains OM5, OM4, OM3, and OM2 of

O. carboxidovorans are very similar as indicated by a close immunological relationship, same molecular masses, same subunit structure, same types and number of cofactors, indistinguishable electron acceptor specificity, and co-migration upon non-denaturing or SDS-PAGE (Cypionka et al., 1980; Meyer and Rohde, 1984; Hugendieck and Meyer, 1992). The CO dehydrogenase from *O. carboxidovorans* is a 277-kDa Mo- and Cu-containing iron-sulfur flavoprotein (Meyer et al., 2000); therefore, growth with CO has a special requirement for Mo and Cu. The enzyme contains the cofactor molybdopterin cytosine dinucleotide. CO dehydrogenase has been crystallized in different states and structurally characterized (Gremer et al., 2000; Meyer et al., 2000; Dobbek, et al., 2002). The bimetallic [CuSMoO₂] active site of the enzyme has also been studied by x-ray spectroscopy (Gnida et al., 2003). The subunit structure of CO dehydrogenase is (LMS)₂, and it consists of a dimer of LMS heterotrimers. Each heterotrimer is composed of a 17.8-kDa iron-sulfur protein (S, 166 amino acid residues), which carries two types of [2Fe-2S] clusters, a 30.2-kDa flavoprotein (M, 288 amino acid residues), which contains a noncovalently bound FAD cofactor, and a 88.7-kDa molybdoprotein (L, amino acid 809 residues), which harbors a [CuSMoO₂] cluster in the active site of the enzyme.

The CO dehydrogenase structural genes *coxMSL* are an integral part of an elaborate CO oxidizing system (Santiago et al., 1999), which itself is part of an extended chemolithoautotrophy module covering 39% of the entire sequence of the 133,056-kb plasmid pHCG3 of *O. carboxidovorans* OM5 Fuhrmann et al., 2003. The respiratory chain of *O. carboxidovorans*, particularly the terminal cytochrome oxidase, is insensitive to CO (Cypionka and Meyer, 1983a).

H₂ oxidation by *O. carboxidovorans* strain OM5 is catalyzed by a membrane-bound hydrogenase [H₂ → 2 H⁺ + 2 e⁻], which has been solubilized, isolated, and characterized (Santiago and Meyer, 1997). It belongs to the class I of hydrogenases and is a heterodimeric 101,692-Da NiFe-protein composed of the polypeptides HoxL and HoxS. The hydrogenase structural genes *hoxLS* are part of the chemolithoautotrophy module on pHCG3. HoxL comprises 604 amino acid residues and has a molecular mass of 67,163 Da. Pre-HoxS comprises 360 amino acid residues and is synthesized as a precursor protein that is cleaved after alanine at position 45, thus producing a mature HoxS of 33,767 Da. The leader sequence corresponds to the signal peptide of small subunits of hydrogenases. The hydropathy plots of HoxL and HoxS indicate the absence of transmembrane helices. Total DNA of species of *Oligotropha* did not hybridize with a *nifH* probe, indicating the absence of genes coding for the conventional, molybdenum-containing nitrogenase (Auling et al., 1988). In addition, growth with N₂ as a sole nitrogen source could not be demonstrated.

Megaplastids have been identified in the strains OM5 (pHCG3: 133 kb) OM4 (pHCG5-a: 158 kb; pHCG5-b: 128 kb), and OM2 (pHCG4: 128 kb) of *O. carboxidovorans* (Kraut and Meyer, 1988). *O. carboxidovorans* strain OM3 does not carry a plasmid. *O. carboxidovorans* OM5 harbors the low copy number 133,058 bp circular DNA plasmid pHCG3, which has been sequenced and is required for the chemolithoautotrophic utilization of CO, H₂, and CO₂ by the bacterium. The calculated G + C content of the plasmid is 60.55 mol%. Loss of the plasmid is associated with loss of the ability to grow with CO or H₂ or to assimilate CO₂. The complete nucleotide sequence of the plasmid pHCG3 was obtained. Sequence analysis indicated 128 open reading frames. Of these, 95 are putative structural genes. The

most striking feature is the occurrence of the four gene clusters *cox*, *cbb*, *hox*, and *tra/trb*, which have functions in the utilization of CO, CO₂ or H₂, and the conjugal transfer of the plasmid, respectively. The clusters *cox*, *cbb*, and *hox* form a 51.2-kb chemolithoautotrophy module, containing 12 *cox* genes (14.54 kb), 13 *cbb* genes (13.33 kb), and 20 *hox* genes (23.35 kb). The 24.58-kb *tra/trb* cluster is separated from the chemolithoautotrophy module by two regions of 25.4- and 29.6-kb with miscellaneous or so far unknown functions. The *tra/trb* cluster carries 10 *tra* genes and 10 *trb* genes, in an arrangement very similar to the Ti-plasmid conjugal transfer system of *Agrobacterium tumefaciens*. The 25.4- and 29.6-kb regions with other functions carry a number of single genes coding for the replication and stabilization of the megaplastid. Among these are an *oriV* coding for the replication proteins RepA, B, and C, two ORFs coding for transposase A, and a single ORF encoding transposase B. The plasmid pHCG3 also contains the insertion sequence SC1190 of *Sulfolobus solfataricus*, a gene function involved in the biosynthesis of acylated homoserine lactones, which participate in quorum sensing, and *rspD* and *rspE*, which function in rhizobium secretion. Most interestingly, the megaplastid pHCG3 of *O. carboxidovorans* carries numerous genes with highest homologies to *Bradyrhizobium japonicum*, *Nitrobacter vulgaris*, *Agrobacterium tumefaciens*, *Rhizobium leguminosarum*, *Rhizobium rhizogenes*, *Rhodobacter capsulatus*, and *Rhodobacter sphaeroides*, which might be taken as an indication of horizontal gene transfer among the members of the α2 and the α3 subclasses of the *Alphaproteobacteria* (Fig. BXII.α.185).

As are all carboxidotrophic bacteria, the members of *Oligotropha* are cosmopolites inhabiting all sorts of environments, including sewage, water, and soil (Meyer and Schlegel, 1978, 1983). Natural enrichment can be found in the covering soil of smoldering charcoal piles (Meyer et al., 1991; Meyer, 1997).

ENRICHMENT AND ISOLATION PROCEDURES

Enrichment and isolation of pure cultures of *Oligotropha* and other aerobic carbon monoxide oxidizing microorganisms from natural habitats apply conditions selective for the ability to proliferate in a mineral medium with CO as a sole energy source and CO₂ as a sole carbon source under respiratory conditions with O₂ as the terminal electron acceptor. The CO present is counterselective for most contaminating microorganisms that cannot utilize CO. Alternatively, enrichments may be carried out under anoxic conditions in the presence of a suitable electron acceptor. Typically, enrichments are done in liquid batch cultures employing a mineral medium (e.g., that of Meyer and Schlegel, 1983, plus the indicated trace elements) incubated in desiccators supplied with a gas atmosphere composed of 5% CO₂, 10% O₂, and 85% CO (alternatively 5% CO₂, 45% CO, and 50% air). The concentrations of CO and O₂ can be varied to modify the selective conditions. About 1 g of environmental sample is suspended in 15 ml of sterile mineral medium contained in 100 ml Erlenmeyer flasks. The flasks are placed in desiccators and the appropriate gas atmosphere is established by evacuation and refilling. After about 1 month with or without shaking at 30°C in the dark, subcultures are made (10% inoculum). Further subcultures are prepared about every 2 weeks (5% inoculum). Aliquots of the suspensions are streaked on agar plates made of mineral medium supplied with nutrient broth and pyruvate (0.1% of each) and solidified with 1.4% agar. The plates are incubated in desiccators at 30°C under the same CO-containing atmosphere used for enrichment. The very fast growing colonies are generally CO-tolerant contaminants. The slow growing colonies appearing after

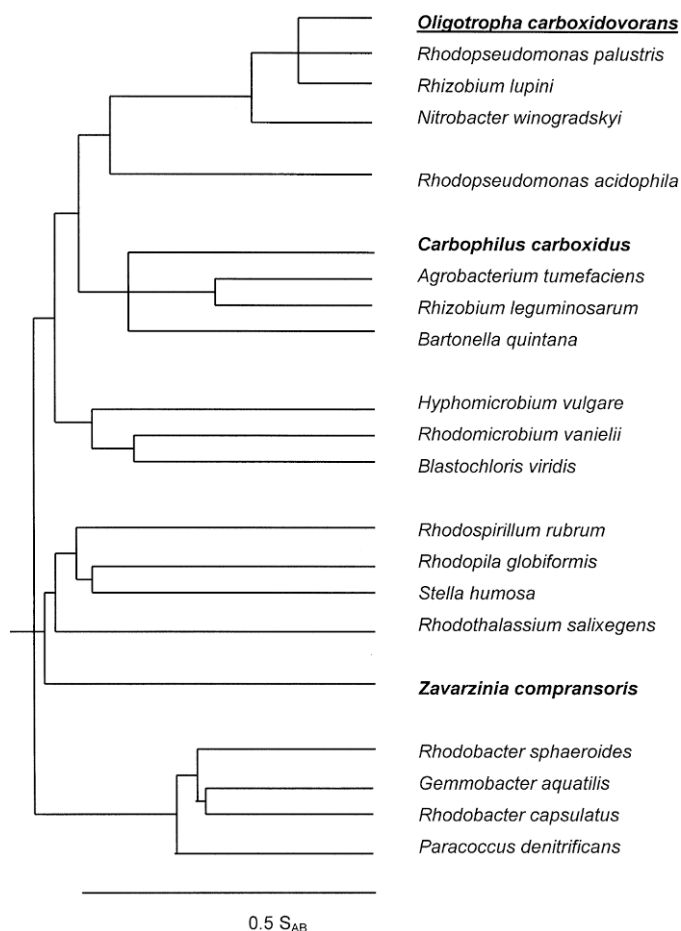


FIGURE BXII.α.185. Position of *Oligotropha carboxidovorans* strain OM5^T (DSM 1227, ATCC 49405) relative to other carboxidotrophic bacteria (*Carboxiphilus carboxidus* DSM 1086^T, *Zavarzinia compransoris* DSM 1231^T) in the phylogenetic tree of the *Alphaproteobacteria*. For a list of similarity coefficients refer to Table 6 of Auling et al. (1988). Modified from Auling et al. (1988). Refer to Meyer et al. (1993) for the transfer and amended descriptions of “*Pseudomonas carboxydovorans*” OM5^T to *Oligotropha*, gen. nov., as *Oligotropha carboxidovorans*, comb. nov., of “*Alcaligenes carboxydus*” DSM 1086^T to *Carboxiphilus*, gen. nov., as *Carboxiphilus carboxidus*, comb. nov., and of “*Pseudomonas compransoris*” DSM 1231^T to *Zavarzinia*, gen. nov., as *Zavarzinia compransoris*, comb. nov. Note that *O. carboxidovorans*, *Bradyrhizobium japonicum* strain 110spc4 (Lorite et al., 2000), *Carboxiphilus carboxidus*, and *Zavarzinia compransoris* are carboxidotrophic, i.e., they contain the molybdo- and copper-enzyme CO dehydrogenase and are capable of chemolithoautotrophic growth with CO as a sole energy and CO₂ as a carbon source under aerobic conditions.

1 week are purified by streaking. Because *Oligotropha* can form stellate aggregations, a pure culture will yield colonies of different diameter and dissimilar appearance. However, the microscopic observation of a single morphological type of cell in small and large colonies along with the formation of aggregates can be taken as evidence that a culture is axenic. Purity of a culture can be further confirmed by determining the viable cell numbers on different media (e.g., plates made up with the salts of pyruvate, lactate, acetate, succinate, or with nutrient broth, with sugars, or with mineral medium in the presence of 5% CO₂, 10% O₂, and 85% CO or 5% CO₂, 10% O₂, and 85% H₂). Pure cultures should reveal either identical colony numbers or no colonies at all. Because of the slow growth on CO or H₂ on solid media, the use of low agar concentrations (1.2–1.4%) is advisable.

DIFFERENTIATION OF THE GENUS *OLIGOTROPHA* FROM OTHER GENERA

Within the *Alphaproteobacteria*, the ability to form CO dehydrogenase and to utilize CO under aerobic chemolithoautotrophic conditions is known from individual strains of the genera *Oligotropha*, *Bradyrhizobium*, *Carboxiphilus*, and *Zavarzinia*. The genus *Oligotropha* is differentiated from *Bradyrhizobium* by the inability to nodulate leguminous plants, the absence of nitrogenase, and the inability to fix N₂. In addition, the variety of carbon sources utilized by the *Bradyrhizobium* species includes a much wider range than those utilized by *Oligotropha*. The genus *Carboxiphilus* is placed by 16S rRNA cataloging in an individual subline of descent within the α2 subclass of the *Alphaproteobacteria* (Fig. BXII.α.185). In contrast to *Oligotropha carboxidovorans*, *Carboxiphilus carboxidus* carries up to five peritrichous flagella, is unable to grow at the expense of H₂ plus CO₂ under aerobic chemolithoautotrophic conditions, and utilizes a broad spectrum of organic compounds, particularly sugars, as source of carbon and energy. In addition, *Carboxiphilus* and *Oligotropha* can be differentiated based on their sensitivity to different kinds of antibiotics and profiles of fatty acids and polyamines (see Table 1 of Meyer et al., 1993). The genus *Zavarzinia* has an isolated phylogenetic position intermediate between the α1 and α2 subclasses of the *Alphaproteobacteria* (Fig. BXII.α.185). *Zavarzinia compransoris* can be differentiated from *Oligotropha* by a very long polar flagellum, the requirement of thiamine as a growth factor, a higher G + C content of the DNA, different sensitivity to antibiotics, and different profiles of fatty acids and polyamines (see Table 1 of Meyer et al., 1993).

TAXONOMIC COMMENTS

On the basis of 16S rRNA cataloging, the presence of signature oligonucleotides in their 16S rRNA catalogues [see Table 5 of Auling et al. (1988)], and the presence of ubiquinone Q-10 as the major quinone (see Table 3 of Auling et al., 1988), sym-homospermidine as the major polyamine (see Table 4 of Auling et al., 1988), and *cis*-vaccinic acid as the major fatty acid (see Table 3 of Auling et al., 1988), *O. carboxidovorans* has been allocated to the class *Alphaproteobacteria* in the phylum *Proteobacteria* (Fig. BXII.α.185). For a dendrogram of relationship, derived from the S_{AB} values by average linkage clustering, refer to Auling et al. (1988). The phylogenetic position of *O. carboxidovorans* indicates a high relationship to certain members of the *Alphaproteobacteria*, such as *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Rhizobium lupini*, *Nitrobacter winogradskyi*, and *N. hamburgensis*.

The type species was formerly known as “*Pseudomonas carboxydovorans*” (Meyer and Schlegel, 1978) and was a re-isolate of “*Hydrogenomonas carboxydovorans*” (Kistner, 1953, 1954), which was lost or lost its properties (see discussion in Meyer and Schlegel, 1978).

Although they have been placed in a separate genus, the strains of *O. carboxidovorans* are phylogenetically related to the dinitrogen fixers *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* (Fig. BXII.α.185). Indeed, *B. japonicum* strain 110spc4 has been demonstrated to grow with CO chemolithoautotrophically and to contain a CO dehydrogenase that is very closely related to the corresponding enzyme from *O. carboxidovorans* strain OM5 (Lorite et al., 2000). However, *O. carboxidovorans* does not fix N₂ and no hybridization with a *nifH* gene probe points to the absence of a conventional nitrogenase.

DNA base ratios of total DNA from *O. carboxidovorans* strains OM5 (62.8 mol% G + C), OM4 (62.6 mol% G + C), OM3 (62.6 mol% G + C), and OM2 (63.1) based on T_m and analysis of the base content by reverse-phase HPLC are narrow (Auling et al., 1988).

List of species of the genus *Oligotropha*

1. ***Oligotropha carboxidovorans*** (Meyer and Schlegel 1978) Meyer, Stackebrandt and Auling 1994, 182^{VP} (Effective publication: Meyer, Stackebrandt and Auling 1993, 391) (*"Pseudomonas carboxydovorans"* Meyer and Schlegel 1978, 42; *"Hydrogenomonas carboxydovorans"* Kistner 1954, 186.) *car.box.i.do'vo.rans*. L. n. *carbo* charcoal, carbon; Gr. adj. *oxy* sour, acid; L. v. *vor* devour; M.L. part. adj. *carboxidovorans* carbon-acid devouring; named for its ability to use CO as a sole carbon and energy source.

So far, only a single species has been identified. The isolates designated OM2, OM3, OM4, and OM5 have all grouped together as strains of the species *Oligotropha carboxidovorans*.

The description is as given for the genus. The strains OM4 and OM5 of *O. carboxidovorans* were isolated from wastewater of the settling pond of the municipal sewage treatment plant of the city of Göttingen (Germany). The strains OM2 and OM3 were isolated from soil.

The mol% G + C of the DNA is: 62.8 (T_m).

Type strain: OM5, ATCC 49405, DSM 1227.

Genus VIII. *Rhodoblastus* Imhoff 2001, 1865^{VP}

JOHANNES F. IMHOFF

Rho.do.blas'tus. Gr. n. *rhodon* the rose; Gr. n. *blastos* bud shoot; M.L. masc. n. *Rhodoblastus* the budding rose.

Cells are rod shaped, motile by means of flagella; show polar growth, budding, and asymmetric cell division. Gram negative and belong to the Alphaproteobacteria. Internal photosynthetic membranes appear as lamellae underlying and parallel to the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids. Straight-chain monounsaturated C_{18:1}, C_{16:1}, and saturated C_{16:0} are the major cellular fatty acids. Contain ubiquinones, rhodoquinones, and menaquinones with 10 isoprene units (Q-10, MK-10, and RQ-10).

Preferred mode of growth is photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth may be possible under anoxic conditions with hydrogen as electron donor. Chemotrophic growth occurs under microoxic to oxic conditions, but with some substrates also anaerobically by fermentation. Growth factors are not required by the type species. **Meso-philic freshwater bacteria with preference for acidic pH.**

Habitat: slightly acidic freshwater ponds.

The mol% G + C of the DNA is: 62.2–66.8.

Type species: Rhodoblastus acidophilus (*Rhodopseudomonas acidophila* Pfennig 1969a, 601) Imhoff 2001, 1865.

FURTHER DESCRIPTIVE INFORMATION

During growth on methanol, *Rhodoblastus acidophilus* assimilates its cell carbon via the ribulosebiphosphate cycle and carboxylation reactions of C₃ fatty acids. There was no evidence of the operation of a reduced C₁ fixation sequence (Sahm et al., 1976). A slow fermentative metabolism may be a property of most *Rhodopseudomonas* species. Gürgün et al. (1976) quantitatively demonstrated the formation from pyruvate of CO₂, formate, acetate, diacetyl, acetoin, and butandiol by *Rhodoblastus acidophilus*. Photoautotrophic growth with hydrogen has been found in *Rhodopseudomonas palustris* in *Rhodoblastus acidophilus* (Pfennig, 1969a). *Rhodoblastus acidophilus* also grows chemoautotrophically with hydrogen and utilizes methanol and formate under these conditions if the oxygen tension is kept at a low level (Siefert and Pfennig, 1979).

In contrast to *Rhodopseudomonas palustris*, sulfate is assimilated by *Rhodoblastus acidophilus* via the adenosine-5'-phosphosulfate

pathway (Imhoff, 1982). Nitrogenase has been found in all species investigated (Madigan et al., 1984). Ammonia assimilation proceeds via glutamine synthetase and glutamate synthase in *Rhodoblastus acidophilus* (NADH-linked) (Brown and Herbert, 1977; Herbert et al., 1978); glutamate dehydrogenase is lacking.

ENRICHMENT AND ISOLATION PROCEDURES

Standard procedures are used for the isolation of purple non-sulfur bacteria (PNSB). A mineral medium that is suitable for most PNSB is also applicable for the cultivation of *Rhodoblastus* species (Imhoff, 1988; Imhoff and Trüper, 1992; for this medium, see the footnote in the chapter describing the genus *Rhodospirillum*). For selective enrichments, the preference for low pH values is used for cultures of *Rhodoblastus acidophilus*. A succinate-mineral medium without growth factors with an initial pH of 5.2 is highly selective for *Rhodoblastus acidophilus* (and also *Rhodomicrobium vannielii*) (Pfennig, 1969a).

MAINTENANCE PROCEDURES

Cultures of *Rhodoblastus* can be preserved in liquid nitrogen, by lyophilization, or at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOBLASTUS* FROM OTHER GENERA

Differential characteristics of the genera and species of *Rhodoblastus*, *Rhodopseudomonas*, *Rhodobium*, *Rhodoplanes*, *Blastochloris*, and *Rhodomicrobium* and are shown in Tables 3 (pp. 125–126) and 4 (p. 127) of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A. Their phylogenetic relationships are shown in Fig. 2 (p. 128) of that chapter. The properties of *Rhodoblastus* are compared to those of *Rhodopseudomonas* species in Tables BXII.α.156 and BXII.α.157.

TAXONOMIC COMMENTS

Rhodoblastus acidophilus contains glucosamine (not 2,3-diamino-2,3-dideoxyhexose) in the lipid A (Tegtmeyer et al., 1985). This is in contrast to *Rhodopseudomonas palustris* and to *Blastochloris* species (also formerly assigned to *Rhodopseudomonas*).

TABLE BXII.α.156. Differential characteristics of the anoxygenic phototrophic purple bacteria belonging to the family *Bradyrhizobiaceae* of the order *Rhizobiales*; genera *Rhodopseudomonas* and *Rhodoblastus*.^a

Characteristic	<i>Rhodopseudomonas palustris</i>	" <i>Rhodopseudomonas cryptolactis</i> "	<i>Rhodopseudomonas julia</i>	<i>Rhodopseudomonas rhenobacensis</i>	<i>Rhodoblastus acidophilus</i>
Cell diameter (μm)	0.6–0.9	1.0	1.0–1.5	0.4–0.6	1.0–1.3
Type of budding	Tube	Sessile	Sessile	Sessile	Sessile
Rosette formation	+	+	+	+	–
Internal membrane system	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae
Motility	+	+	+	+	+
Color of cultures	Brown-red to red	Red	Pink	Red	Red to orange-red
Bacteriochlorophyll	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
Salt requirement	None	None	None	None	None
Optimal pH	6.9	6.8–7.2	6.0–6.5	5.5	5.5–6.0
Optimal temperature	30–37	38–40	25–35	(28)	25–30
Sulfate assimilation	+ (PAPS)	nd	–	+	+ (APS)
Aerobic dark growth	+	+	+	+	+
Denitrification	+ / –	nd	nd	Reduction of nitrate	–
Fermentation of fructose	–	nd	nd	nd	–
Photoautotrophic growth with	H ₂ , thiosulfate, sulfide	–	Sulfide, sulfur	nd	H ₂
Growth factors	<i>p</i> -Aminobenzoic acid, (biotin)	B ₁₂ , niacin, <i>p</i> -aminobenzoic acid	None	<i>p</i> -Aminobenzoic acid	None
<i>Utilization of:</i>					
Benzoate	+	–	–	–	–
Citrate	+	–	–	–	–
Formate	–	–	+	+	–
Glucose	–	–	+	–	–
Tartrate	–	–	–	+	–
Sulfide	+	–	+	nd	–
Thiosulfate	+	–	nd	–	–
Mol % G + C of the DNA	64.8–66.3 (Bd)	68.8	63.5	65.4 (HPLC)	62.2–66.8 (Bd)
Cytochrome <i>c</i> ₂ size	Large	nd	nd	nd	Small
Major quinones	Q-10	nd	nd	Q-10	Q-10, MK-10, RQ-10
<i>Major fatty acids</i>					
C _{14:0}	trace	nd	nd	nd	0.8
C _{16:0}	5.2	nd	nd	11.7	14.8
C _{16:1}	3.1	nd	nd	9.5	37.2
C _{18:0}	7.3	nd	nd	7.8	0.8
C _{18:1}	79.7	nd	nd	66.1	46.0

^aSymbols: +, positive in most strains; –, negative in most strains; + / –, variable in different strains; nd, not determined; (+), weak growth or microaerobic growth only; (APS), via adenosine-5'-phosphosulfate; (PAPS), via 3'-phosphoadenosine-5'-phosphosulfate; (biotin) biotin is required by some strains; Q-10, ubiquinone 10; MK-10, menaquinone 10; RQ-10, rhodoquinone 10. Bd, buoyant density.

List of species of the genus *Rhodoblastus*

- Rhodoblastus acidophilus*** (*Rhodopseudomonas acidophila* Pfennig 1969a, 601^{AL}) Imhoff 2001, 1865^{VP}
a.ci.do'phi.lus. L. adj. *acidus* sour; M.L. neut. n. *acidum* an acid; Gr. adj. *philos* loving; M.L. masc. adj. *acidophilus* acid-loving.

Cells are rod shaped to elongate-ovoid, slightly curved, 1.0–1.3 × 2.0–5.0 μm, motile by polar flagella, and Gram negative. Daughter cells originate by polar growth as sessile buds at the pole opposite that bearing the flagella; there is no tube or filament between mother and daughter cells (Fig. BXII.α.186). When the daughter cell reaches the size of the mother cell, cell division is completed by constriction. In the next cycle, both cells form buds at the poles of the former cell division. Under certain conditions, rosettes and clusters are formed that are similar to those of the type species. In media lacking calcium ions, cells are immotile. Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane. Color of anaerobic liquid cultures is purple-red to orange-brown. Cells grown under oxic conditions are colorless to light pink or orange. Absorption spectra of living cells show maxima at 375, 460, 490, 525, 590, 805, 855, and 890 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esteri-

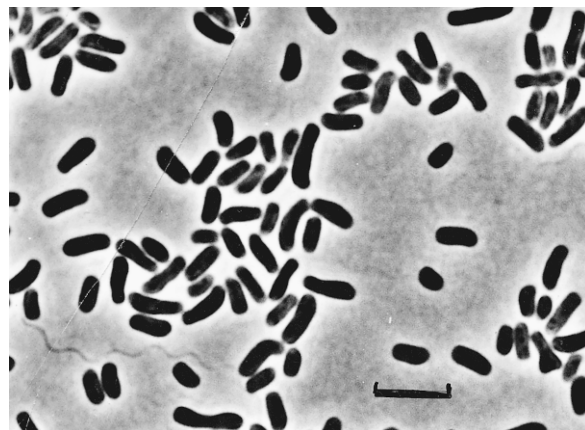


FIGURE BXII.α.186. *Rhodoblastus acidophilus* strain 2751. The shape of some of the cells is indicative of the polar type of cell growth. Tufts of detached flagella can be seen. Phase-contrast micrograph. Bar = 5 μm. (Courtesy of N. Pfennig.)

TABLE BXII.α.157. Growth substrates of the anoxygenic phototrophic purple bacteria belonging to the family *Bradyrhizobiaceae* of the order *Rhizobiales*, genera *Rhodopseudomonas* and *Rhodoblastus*^a

Source/donor	<i>Rhodopseudomonas palustris</i>	<i>"Rhodopseudomonas cryptolactis"</i>	<i>Rhodopseudomonas julia</i>	<i>Rhodopseudomonas rhenobacensis</i>	<i>Rhodoblastus acidophilus</i>
<i>Carbon source</i>					
Acetate	+	+	+	+	+
Aspartate	+ / -	-	+	nd	-
Benzoate	+	-	-	-	-
Butyrate	+	nd	+	+	+ / -
Caproate	+	nd	-	nd	+ / -
Caprylate	+	nd	-	nd	-
Citrate	+ / -	nd	-	-	+ / -
Ethanol	+ / -	nd	-	+	+
Formate	+	nd	+	+	+ / -
D-Fructose	+ / -	-	+	-	-
Fumarate	+	nd	+	+	+
D-Glucose	+ / -	-	+	-	+ / -
Glutamate	+	-	-	-	-
Glycerol	+	nd	+	nd	+ / -
Glycolate	+	nd	nd	nd	+ / -
Lactate	+	+	nd	+	+
Malate	+	+	+	+	+
Malonate	+	nd	-	nd	+ / -
Mannitol	+ / -	nd	+	nd	-
Methanol	+ / -	nd	-	-	+ / -
Propanol	+	nd	-	nd	nd
Propionate	+	nd	+	-	+
Pyruvate	+	+	+	+	+
Sorbitol	+	nd	+	nd	+
Succinate	+	+	+	+	+
Tartrate	-	nd	-	+	+ / -
Valerate	+	nd	+	nd	+
<i>Electron donor</i>					
Sulfide	+	nd	+	nd	-
Thiosulfate	+	nd	nd	nd	-

^aSymbols: +, positive in most strains; -, negative in most strains; + / - variable in different strains; nd, not determined.

fied with phytol) and carotenoids of the spirilloxanthin series with glucosides of rhodopin and rhodopinal. The latter are characteristic of this species.

Photoheterotrophic growth with a number of organic carbon sources is the preferred growth mode. Photoautotrophic growth is possible with hydrogen as electron donor; sulfide and thiosulfate cannot be used. Cells grow under microoxic to oxic conditions in the dark, with hydrogen as electron donor autotrophically. The organic carbon sources used are acetate, propionate, butyrate, lactate, pyruvate, fumarate, malate, succinate, valerate, formate, methanol, and ethanol. Not used are caprylate, pelargonate, glycerol, ben-

zoate, sugars, sugar alcohols, glutamate, and other amino acids. Sulfate can be used as sole sulfur source. Ammonia, dinitrogen, and some amino acids are used as nitrogen source. Growth factors are not required; yeast extract, or other complex nutrients do not increase the growth rate.

Mesophilic freshwater bacterium with optimal growth at 25–30°C and pH 5.5–6.0.

Ubiquinones and menaquinones with 10 isoprene units (Q-10 and MK-10) are present.

The mol% G + C of the DNA is: 62.2–66.8 (Bd).

Deposited strain: Pfennig 7050, ATCC 25092, DSM 137.

GenBank accession number (16S rRNA): M34128.

Genus IX. *Rhodopseudomonas* Czurda and Maresch 1937, 119^{AL} emend. Imhoff, Trüper and Pfennig 1984, 341

JOHANNES F. IMHOFF

Rho.do.pseu.do.mo'nas. Gr. n. *rhodon* the rose; M.L. fem. n. *Pseudomonas* a bacterial genus; M.L. fem. n. *Rhodopseudomonas* the red *Pseudomonas*.

Cells are rod-shaped, motile by means of polar or subpolar flagella; show polar growth, budding and asymmetric cell division. Gram-negative and belong to the *Alphaproteobacteria*. Internal photosynthetic membranes appear as lamellae underlying and parallel to the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Straight-chain monounsaturated C_{18:1} is the major component of cellular fatty acids. Contains ubiquinones with 10 isoprene units (Q-10).

Preferred mode of growth is photoheterotrophic under anoxic conditions in the light. Photoautotrophic growth may be possible under anoxic conditions with hydrogen, thiosulfate, or sulfide as electron donor. Chemotrophic growth under microoxic to oxic conditions is also possible. Growth factors may be required.

Mesophilic freshwater bacteria with preference for neutral pH.

The mol% G + C of the DNA is: 64.8–66.3.

Type species: Rhodopseudomonas palustris (Molisch 1907) van Niel 1944, 89 (*"Rhodobacillus palustris"* Molisch 1907, 14.)

FURTHER DESCRIPTIVE INFORMATION

Rhodopseudomonas species are characterized by asymmetric cells during cell division. Most characteristic is the formation of prosthecae and rosette-like cell aggregates.

A reductive and oxygen-inhibited degradation of benzoate has been demonstrated in *R. palustris* (Dutton and Evans, 1969, 1978; Gibson and Harwood, 1995). Citrate may be used by some strains of *Rhodopseudomonas palustris*. During fermentation, pyruvate is quantitatively transformed into CO₂, formate, acetate, lactate, butyrate, and acetoin by *R. palustris* (Gürgün et al., 1976). Photoautotrophic growth with hydrogen occurs in *R. palustris* (Klemme, 1968).

Sulfate can serve as sole sulfur source and is assimilated via the 3'-phosphoadenosine-5'-phosphosulfate (Imhoff, 1982). *R. palustris* is able to use reduced sulfur compounds as photosynthetic electron donors. Sulfide is oxidized by *R. palustris* to elemental sulfur (Hansen, 1974), and thiosulfate to sulfate (Rolls and Lindstrom, 1967).

In *R. palustris* (Zumft and Castillo, 1978) nitrogenase shows a "switch-off" effect by ammonia and some organic nitrogen compounds. This effect is reversible. Ammonia assimilation proceeds via glutamine synthetase and glutamate synthase in *Rhodopseudomonas palustris* (NADH-linked) (Brown and Herbert, 1977; Herbert et al., 1978). A glutamate dehydrogenase is present. Besides ammonia, *R. palustris* uses a great number of nitrogen sources: nitrate, dimethylamine, trimethylamine, azaguanine, L-histidine, L-glutamate, L-aspartate, L-arginine, L-cysteine, L-methionine, DL-lysine, DL-alanine, DL-leucine, casein hydrolysate (Malofeeva and Laush, 1976), guanine, uric acid, xanthine (Aretz et al., 1978), cytidine, and cytosine (Kaspari, 1979). Most strains of *R. palustris* are not able to use nitrate as a nitrogen source (Klemme, 1979). Three isolates, however, are capable of dissimilatory nitrate reduction and growth under anoxic dark conditions with nitrate as terminal electron acceptor (Klemme et al., 1980).

Rhodopseudomonas palustris and *Nitrobacter winogradskyi* show a high degree of 16S rDNA sequence similarity (Seewaldt et al., 1982), and the lipid A structure of the lipopolysaccharide of *Nitrobacter* species contains the unusual 2,3-diamino-2,3-deoxy-D-glucose that is characteristic for *Rhodopseudomonas palustris* (Mayer et al., 1983, 1984). This sugar has also been found in *Blastochloris viridis* (see Weckesser et al., 1979), *Blastochloris sulfoviridis* (Ahamed et al., 1982), *Pseudomonas diminuta*, and *Pseudomonas vesicularis* (Mayer et al., 1983).

ENRICHMENT AND ISOLATION PROCEDURES

Rhodopseudomonas palustris is a very common species of purple nonsulfur bacteria (PNSB) in nature, and many enrichments select for *R. palustris*. Benzoate is a carbon source particularly for enrichment of this species. Standard procedures for other PNSB can be used for isolation of *Rhodopseudomonas* species. A mineral medium that is suitable for most PNSB can be applied for isolation and cultivation of *Rhodopseudomonas* species (Imhoff, 1988; Imhoff and Trüper, 1992; see chapter Genus *Rhodospirillum* for this medium).

MAINTENANCE PROCEDURES

Cultures of *Rhodopseudomonas* species are well preserved in liquid nitrogen, by lyophilization or at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOPSEUDOMONAS* FROM OTHER GENERA

Differential characteristics of the genera and species of *Rhodopseudomonas*, *Rhodobium*, *Rhodoplanes*, *Blastochloris*, *Rhodoblastus*, and *Rhodomicrobium* are shown in Tables 3 and 4 of the introductory chapter "Anoxygenic Phototrophic Purple Bacteria." (pp. 125–127, Volume 2, Part A). Their phylogenetic relationships are shown in Fig. 2 (p. 128) of that same chapter.

TAXONOMIC COMMENTS

After the removal of PNSB that contained vesicular internal photosynthetic membranes and those that were *Betaproteobacteria* from the genus *Rhodopseudomonas* (Imhoff et al., 1984), those that remained had lamellar internal membrane structures and a budding mode of growth and reproduction. Even after the removal of bacteria now recognized as species of *Rhodopila*, *Rhodobacter*, *Rhodovulum*, and *Rubrivivax*, the genus *Rhodopseudomonas* still represented a heterogeneous assemblage of species (Imhoff et al., 1984). Based on 16S rDNA sequence data (Kawasaki et al., 1993b), isolation and description of new species, and additional data, *Rhodopseudomonas rosea* was transferred to *Rhodoplanes roseus* (Hiraishi and Ueda, 1994b), *Rhodopseudomonas marina* to the new genus *Rhodobium* as *Rhodobium marinum*, and *R. viridis* and *R. sulfoviridis* to the genus *Blastochloris* as *Blastochloris viridis* and *Blastochloris sulfoviridis* (Hiraishi, 1997). *Rhodopseudomonas acidophila* was transferred to *Rhodoblastus acidophilus* (Imhoff, 2001).

Rhodopseudomonas blastica has many chemotaxonomic properties in common with bacteria in the informal group "alpha-3 proteobacteria", *Rhodobacter*, *Rhodovulum*, and *Paracoccus denitrificans*. Its 16S rDNA sequence is most similar to and clusters with the *Rhodobacter* species. Therefore, it was transferred to this genus and is now known as *Rhodobacter blasticus* (Kawasaki et al., 1993b).

Hiraishi et al. (1992b) considered *Rhodopseudomonas rutila* as identical to *R. palustris* and the species name as a later subjective synonym of *R. palustris*, although it has been described as a separate species (Akiba et al., 1983). In the original species description *R. palustris* was not included as a reference organism and properties of the new isolates were reinvestigated later (Hiraishi et al., 1992b). These authors established identity between the type strains of *R. palustris* and *R. rutila*. In particular, in regard to the utilization of acetate, propionate, benzoate, fructose, sulfide, and thiosulfate, Hiraishi et al. (1992b) disagreed with Akiba et al. (1983) and found conformity with the properties of six *R. palustris* strains. The former authors also found that *Rhodopseudomonas rutila* requires *p*-aminobenzoic acid as a growth factor. Both bacteria were identical in quinone, lipid and fatty acid composition and 16S rDNA sequence (accession number of the 16S rDNA sequence of strain ATCC 33872, the former type of *R. rutila* at EMBL is D14435). DNA–DNA similarity between *R. rutila* and *R. palustris* strains was between 80–90%.

In addition to *R. palustris*, three species of *Rhodopseudomonas* are known. *Rhodopseudomonas rhenobacensis* is a recently described bacterium notably reducing nitrate (Hougardy et al., 2000). *Rhodopseudomonas julia* is an acidic sulfur spring isolate with the distinct ability to grow photoautotrophically (Kompantseva, 1989a). "*Rhodopseudomonas cryptolactis*" was also isolated from a hot spring and is characterized by a lack of autotrophic growth and a quite restricted substrate spectrum (Stadtwaldedemchick et al., 1990). This bacterium has not been validly published.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOPSEUDOMONAS*

Characters that differentiate the species of the genus *Rhodopseudomonas* from each other and from *Rhodoblastus acidophilus*

are given in Tables BXII.α.156 and BXII.α.157 of the chapter on *Rhodoblastus*.

List of species of the genus Rhodopseudomonas

1. ***Rhodopseudomonas palustris*** (Molisch 1907) van Niel 1944, 89^{AL} (*"Rhodobacillus palustris"* Molisch 1907, 14.) *pa.hus' tris*. L. fem. adj. *palustris* marshy, swampy.

Individual cells are rod-shaped to ovoid, occasionally slightly curved, $0.6\text{--}0.9 \times 1.2\text{--}2.0\text{ }\mu\text{m}$, motile by means of subpolar flagella, and reproduce by budding. The mother cell produces a slender prostheca 1.5–2.0 times the length of the original cell at the pole opposite to that bearing the flagella. The end of the prostheca swells, and the daughter cell grows, producing a dumbbell-shaped organism (Fig. BXII.α.187). Asymmetric division then takes place. Young individual cells are highly motile. The formation of rosettes and clusters in which the individual cells are attached to each other at their flagellated poles are characteristic in older cultures. In certain complex media, individual cells become up to 10 μm long and irregular in shape. Internal photosynthetic membranes appear as lamellae underlying and parallel to the cytoplasmic membrane; no lamellae are present in the prostheca. Color of cell suspensions is red to brownish-red. Living cells show absorption maxima at 375, 468, 493, 520–545, 589, 802, and 860–875 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the normal spirilloxanthin series.

Photoautotrophic growth occurs with hydrogen, sulfide, and thiosulfate as electron donors in the presence of small amounts of yeast extract. Photoheterotrophic growth is possible with various organic substrates. Chemotrophic growth occurs in the dark under microoxic to oxic conditions. With some substrates fermentation takes place under anoxic conditions. Sulfate can be used as sole sulfur source. The nitrogen source may be ammonia, dinitrogen, some amino acids, or for a few strains, nitrate.

Growth factors required are *p*-aminobenzoate and, for

some strains, biotin; yeast extract stimulates growth considerably.

Ubiquinones with 10 isoprene units (Q-10) are present. Mesophilic freshwater bacteria with optimal growth at 30–37°C and pH 6.9 (pH range: 5.5–8.5).

The mol% G + C of the DNA is: 64.8–66.3 (Bd).

Type strain: ATH 2.1.6, ATCC 17001, DSM 123.

GenBank accession number (16S rRNA): D12700, D25312, L11664.

2. ***Rhodopseudomonas julia*** Kompantseva 1993, 188^{VP} (Effective publication: Kompantseva 1989a, 258.)

ju.li.a. L. fem. adj. *julia* discovered and described in July.

Cells are straight or slightly curved rods, $1\text{--}1.5 \times 2.5\text{ }\mu\text{m}$. Formation of rosettes is characteristic. Propagation by budding; the bud is sessile. Motile by means of polar flagella. Photosynthetic membrane systems appear as concentric lamellae. Color of cell suspensions is pink to purple. The absorption spectra of living cell suspensions may have maxima at 380, 590, 803, and 850 nm (indicative of bacteriochlorophyll *a*) and at 490, 516, and 550 nm (indicating the presence of carotenoids of the normal spirilloxanthin series).

Facultative photoorganoheterotrophs. Can also grow chemoorganotrophically under microaerobic conditions and photolithoautotrophically, oxidizing H_2S or S^0 to sulfate. The intermediate product of H_2S oxidation is elemental sulfur, which is deposited both outside and inside the cells. Ammonia and Casamino acids, but not nitrate, are utilized as nitrogen sources; H_2S , S^0 , cysteine, and glutathione, but not sulfate, serve as sulfur sources. No vitamins are required. Hydrogen donors and carbon sources: fatty acids to valerate, hydroxy acids of the tricarboxylic and acid cycle, formate, pyruvate, aspartate, glycerol, glucose, gluconate, mannitol, sorbitol, fructose, yeast extract, and Casamino acids. Substrates not utilized: arginine, benzoate, glutamate, malonate, tartrate, citrate, and alcohols. Storage material: poly- β -hydroxybutyrate. Catalase activity is present.

Optimal growth conditions: pH 6.0–6.5, 25–35°C, light intensity more than 2000 lux.

Habitat: slightly and moderately acid sulfide springs having a high content of elemental sulfur.

The mol% G + C of the DNA is: 63.5 ± 1 (method not available in the literature).

Type strain: KR-11-67, ATCC 52215, DSM 11549.

GenBank accession number (16S rRNA): AB087720.

3. ***Rhodopseudomonas rhenobacensis*** Hougardy, Tindall, Klemme 2000, 991^{VP}

rhe.no.ba.cen'sis. M.L. adj. *rhénobacensis* pertaining to Rheinbach, a small German town.

Cells are rods, $0.4\text{--}0.6 \times 1.5\text{--}2\text{ }\mu\text{m}$. Multiply by budding and form characteristic rosette-like aggregates. Motile by means of polar flagella. Photosynthetic membrane system appears as lamellae. Color of cell suspensions is red. The absorption spectra of living cell suspensions show maxima at 376, 591, 805, and 878 nm (bacteriochlorophyll *a*) and at 471, 503, and 540 nm (carotenoids).

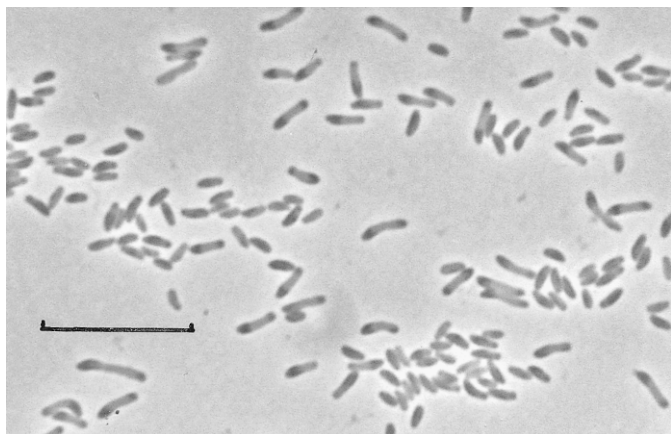


FIGURE BXII.α.187. *Rhodopseudomonas palustris* strain 1850 grown in succinate-yeast extract medium. The budding type of reproduction can be recognized in a number of cells. Phase-contrast micrograph. Bar = 10 μm . (Courtesy of N. Pfennig.)

Facultative photoorganoheterotrophs. Also grow chemoorganotrophically under aerobic conditions. Nitrate is reduced to nitrite, which accumulates in the medium. Hydrogen donors and carbon sources: formate, acetate, pyruvate, lactate, malate, succinate, fumarate, tartrate, gluconate, ethanol, and butyrate (plus carbonate). Substrates not utilized: citrate, benzoate, glucose, fructose, methanol, propionate, arginine, and glutamate. Gelatin is not hydrolyzed. Ammonia, glutamate dinitrogen, and yeast extract serve as nitrogen sources. *p*-Aminobenzoic acid required as growth factor.

Optimal pH 5.5 (range: 5.0–8.0). NaCl is not required and completely inhibits growth at 1%.

Habitat: freshwater lake sediment.

The mol% G + C of the DNA is: 65.4 (HPLC).

Type strain: Rb, DSM 12706.

GenBank accession number (16S rRNA): AJ132402.

4. “*Rhodopseudomonas cryptolactis*”

cryp.to' lac.tis. Gr. adj. *cryptos* hidden; L. fem. n. *lactes* milk; M.L. fem. adj. *cryptolactis* hidden of milk.

Cells are rod-shaped, approximately $1.0 \times 4 \mu\text{m}$, motile by means of flagella, and reproduce by budding. The formation of rosettes is observed. Internal photosynthetic membranes appear as lamellae underlying the cytoplasmic membrane. Color of cell suspensions is red. Absorption maxima of living cells at 590, 800, and 857 nm is indicative of the presence of bacteriochlorophyll *a*.

Photoheterotrophic growth occurs with pyruvate, acetate, malate, succinate, and lactate (only in the presence of bicarbonate). Hydrogen and sulfide are not used as electron donors. Chemoheterotrophic growth occurs in the dark under oxic conditions. Ammonia, dinitrogen, urea, and L-glutamine are used as nitrogen source. Growth factors required are vitamin B₁₂, niacin, and *p*-aminobenzoate.

Mesophilic freshwater bacterium with optimal growth at 40°C (range: 35–46°C), and pH 6.8–7.2 (pH range: 6.4–8.5).

Habitat: thermopolis Hot Spring, Wyoming, USA.

The mol% G + C of the DNA is: 68.8.

Deposited strain: ATCC 49414.

Family VIII. *Hyphomicrobiaceae* Babudieri 1950, 589

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Hy.pho.mi.cro.bi.a' ce.ae. M.L. neut. n. *Hyphomicrobium* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Hyphomicrobiaceae* the *Hyphomicrobium* family.

The family *Hyphomicrobiaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Hyphomicrobium* (type genus), *Anca-lomicrobium*, *Ancylobacter*, *Angulomicrobium*, *Aquabacter*, *Azorhizobium*, *Blastochloris*, *Devosia*, *Dichotomicrobium*, *Filomicrobium*, *Gemmiger*, *Labrys*, *Methylorhabdus*, *Pedomicrobium*, *Prosthecomicrobium*, *Rhodomicrobium*, *Rhodoplanes*, *Seliberia*, *Starkeya*, and *Xanthobacter*.

The family is morphologically, metabolically, and ecologically diverse. Many members form hyphae or prosthecae; a number reproduce by budding. Includes organisms that are photosynthetic, facultatively methylotrophic, facultatively chemolithoautotrophic, and chemoheterotrophic.

Type genus: *Hyphomicrobium* Stutzer and Hartleb 1898, 76.

Genus I. *Hyphomicrobium* Stutzer and Hartleb 1898, 76^{AL}

CHRISTIAN GLIESCHE, ANDREAS FESEFELDT AND PETER HIRSCH

Hy.pho.mi.cro' bi.um. Gr. *hyphe* thread; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. neut. n. *Hyphomicrobium* thread-producing microbe.

Cells 0.3–1.2 \times 1–3 μm ; rod-shaped with pointed ends, or **oval, egg-, or bean-shaped**; produce **monopolar or bipolar filamentous outgrowths** (hyphae or prosthecae) of varying length and 0.2–0.3 μm in diameter. Hyphae are not septate, but hyphal cytoplasmic membranes show conspicuous constrictions. **Hyphae may be truly branched**; secondary branches are rare. Cells stain with carbol fuchsin, but stain weakly with aqueous aniline dyes. Gram negative and non-acid-fast. Do not form spores.

Multiplication: **daughter cell formation by a budding process** at one hyphal tip at a time (Figs. BXII.α.188 and BXII.α.189); mature buds become **motile swimmers** that break off and may attach to surfaces or other cells to form clumps or **rosettes**. Motility is lost soon after swarmer cell liberation and/or attachment. Older cultures may lack motile swarmer cells. **Poly-β-hydroxybutyrate is stored by most cells, usually at a distinct cell pole**.

Colonies on solid media are small, even after prolonged incubation; they are **brownish** in transmitted light and **bright beige or colorless** in reflected light. Colony surface is shiny or granular,

folded or smooth. Older colonies often display concentric rings and change color to darker brown or bright yellow-orange.

Chemoorganotrophic, aerobic. Carbon dioxide is required for growth. **Oligocarboophilic**, i.e., growth can occur on mineral salts media without carbon sources added to the medium, possibly instead resulting from the presence of volatile carbon and energy sources. Growth may be stimulated by soil extract if the pH remains near neutral. **Good growth with 0.1–0.2% (w/v) of one-carbon compounds, such as methanol, methylamine, or even chloromethane**. NH₄⁺ is a good nitrogen source, but organic nitrogen compounds (some amino acids) may also be utilized. No nitrification. Widely distributed in soils and aquatic habitats. Mesophilic. Optimal pH: above 7.0, except for one species with a lower optimal pH.

The mol% G + C of the DNA is: 59–65 (*T_m*).

Type species: *Hyphomicrobium vulgare* Stutzer and Hartleb 1898, 76.



FIGURE BXII.α.188. *Hyphomicrobium facile* IFAM B-522. Mother cell with hyphae and young bud. Bar = 1.0 μm.



FIGURE BXII.α.189. *Hyphomicrobium facile* subsp. *ureaphilum* IFAM CO-582. Mother cell, hyphae, and mature bud. The mother cell contains a storage granule (poly-β-hydroxybutyrate). Shadow-cast. Bar = 1 μm.

FURTHER DESCRIPTIVE INFORMATION

Phylogeny and probes Analysis of 5S rDNA (Stackebrandt et al., 1988a; Boulygina et al., 1993), 16S rRNA (Stackebrandt et al., 1988a), and 16S rDNA (Stackebrandt et al., 1988a; Tsuji et al., 1990; Tuhela et al., 1997; Rainey et al., 1998; Borodina et al., 2000; De Marco et al., 2000; Layton et al., 2000; McDonald et al., 2001), determination of 16S rRNA cistron similarities (Moore

1977; Roggentin and Hirsch 1989), membrane fatty acids and quinones (Urakami and Komagata 1979, 1986b, 1987a, b; Sittig and Hirsch, 1992) have confirmed the membership of hyphomicrobia in the Class *Alphaproteobacteria* (Order *Rhizobiales*). The coherency of the genus is supported by phage typing (Gliesche et al., 1988), low-molecular-mass RNA patterns (Höfle, 1990), and enzyme electrophoresis patterns (Urakami and Komagata,

1981). However, 16S rRNA cataloguing (Stackebrandt et al., 1988a) and 16S rDNA sequencing (Rainey et al., 1998; Borodina et al., 2000; Layton et al., 2000) show the genus *Filomicrobium* to branch within the radiation of *Hyphomicrobium*. Furthermore, 16S rRNA cistron similarities (Roggentin and Hirsch, 1989) and 16S rDNA sequences (Cox and Sly, 1997; Layton et al., 2000) confirm a close relationship of hyphomicrobia to the genus *Pedomicrobium*.

Based on data on morphological and physiological characteristics, 5S rRNA sequences, fatty acid and lipid composition of their membranes, and sensitivity to antibiotics, an evolutionary pathway from *Caulobacter* to *Hyphomicrobium* has been proposed by Nikitin et al. (1990). However, this phylogenetic view is not supported by 16S rDNA sequence analysis (Rainey et al., 1998; Sly et al., 1999; Layton et al., 2000).

The intragenetic structure based on 16S rDNA sequences shows two clusters (Rainey et al., 1998). Cluster I contains the neotype species of *H. vulgare*, as well as *H. aestuarii*, *H. hollandicum*, and *H. zavarzinii*. Cluster II comprises *H. facile*, *H. denitrificans*, *H. methylovorum*, and *H. chloromethanicum* (McDonald et al., 2001). 16S rDNA signature nucleotides that define clusters I and II of *Hyphomicrobium* species are given by Rainey et al. (1998). A detailed phylogenetic investigation of the 16S rDNA of hyphomicrobia isolated from a sewage treatment plant and its receiving lake (Holm et al., 1996) has revealed a significantly greater diversity and resulted in the creation of additional clusters, III and IV. Of these, cluster III comprises 16 strains (B 376, P 251, P 262, P 139, P 165, P 482, B 47, P 425, P 148, P 37, A 676, IFAM 1460, P 645, B 455, A 739, and A 679). Cluster IV consists of *Hyphomicrobium* sp. 502 and *Filomicrobium fusiforme* (Rainey and Stackebrandt, unpublished data). A dendrogram showing the phylogenetic position of the genera *Hyphomicrobium* and *Filomicrobium* among the closest relatives in the *Alphaproteobacteria* is given in Fig. BXII.α.190. This intragenetic structure is supported by a phylogenetic analysis of a fragment of the gene coding for the α -subunit of methanol dehydrogenase (*mxhF*). Fig. BXII.α.191 shows a dendrogram of hyphomicrobia based on published sequences of this gene fragment. A comparative sequence analysis of this *mxhF* gene fragment, including 150 strains, indicates a coherent evolution of this essential metabolic gene and the 16S rRNA gene in hyphomicrobia. The dendrogram based on the *mxhF* nucleic acid sequence has a finer resolution and

results in the five clusters A to E, which correspond exactly to the 16S rDNA-based subdivision (*mxhF* clusters B and C are identical to the 16S cluster II) (Fesefeldt, 1998). Furthermore, genomic DNA of *Filomicrobium fusiforme* DSM 5304^T gives a strong hybridization signal with a fragment (position 1009–1553, according to *Paracoccus denitrificans* PD1207) of the *mxhF* gene of *Hyphomicrobium* sp. P 502 (*mxhF* cluster E) (Fesefeldt, 1998). This observation supports the close relationship between both genera and indicates that *Filomicrobium* retains parts of the methanol dehydrogenase gene cluster.

The distinctiveness of *Hyphomicrobium* species has been demonstrated by DNA–DNA hybridizations (Moore and Hirsch, 1972; Urakami et al., 1985; Gebers et al., 1986; Doronina et al., 1996b; McDonald et al., 2001); low-molecular-mass RNA patterns (Höfle, 1990); 16S rRNA cistron similarities (Roggentin and Hirsch, 1989); membrane fatty acids, phospholipids, and quinones (Sittig and Hirsch, 1992); and differences of more than 1.2% in 16S rDNA sequences for the closely related species (Rainey et al., 1998). Unfortunately, molecular analyses have not yet been conducted for undescribed strains for which species status has been discussed based on phenotypic analysis (Vedenina et al., 1991; Holm et al., 1996).

Probes for hyphomicrobia have been developed specifically for different taxonomic levels: (1) species-specific (Hfa-1 for *H. facile* [Fesefeldt et al., 1997] and Hvu-1 for *H. vulgare* [Gliesche et al., 1997]); (2) cluster-specific (S-S-HyphoCI-648-a-A-20 for *Hyphomicrobium* Cluster I and S-S-HyphoCII-654-a-A-18 for *Hyphomicrobium* Cluster II [Layton et al., 2000]); and (3) genus-specific (S-G-Hypho-1241-a-A-19 for *Hyphomicrobium* spp. [Layton et al., 2000]). Probe Hvu1034 (Neef et al., 1996) has exhibited an uncertain specificity (Neef and Fesefeldt, unpublished data).

Cell morphology The cell morphology may vary with growth conditions. In media with low nutrient concentrations, hyphae may elongate up to 300 μ m. The degree of branching depends on the type and concentration of carbon source present. Some strains have helically twisted hyphae. Stirring laboratory cultures may result in increased intercalary bud formation in the hyphae. The number and attachment sites of flagella vary among the nine described species. Most hyphomicrobia that utilize C₁ compounds have 1–3 subpolar flagella, which are easily shed.

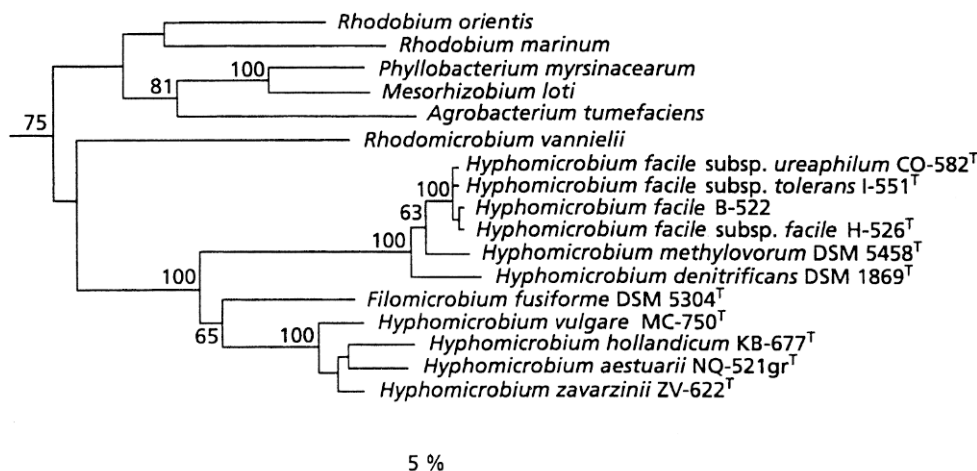


FIGURE BXII.α.190. Dendrogram based on 16S rDNA sequences, showing the phylogenetic position of the genera *Hyphomicrobium* and *Filomicrobium* among the closest relatives in the *Alphaproteobacteria* after Rainey et al. (1998).

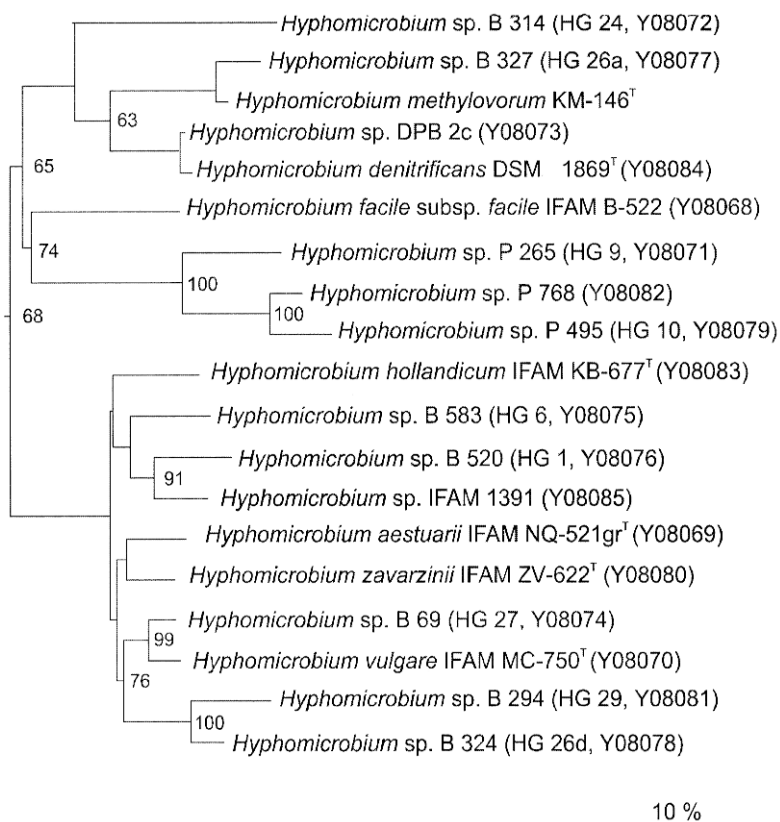


FIGURE BXII.α.191. Phylogenetic relationship constructed for partial *msaF* genes of selected *Hyphomicrobium* strains (based on the nucleotide sequence position 1009–1553 of *Paracoccus denitrificans* PD 1207). The dendrogram was generated by phylogenetic-distance analysis with a neighbor-joining algorithm. The bootstrap values indicate the percentage of 100 replicate trees supporting the branching order. Values below 50 were omitted. DNA–DNA hybridization group and accession numbers are given in brackets. Bar = 10 mutations per 100 sequence positions.

Cell wall and membrane compositions Cell walls of one strain (IFAM B-522) have been analyzed and found to contain α , ϵ -diaminopimelic acid, as well as the other normal components of most Gram-negative walls (Jones and Hirsch, 1968). Mother cells have less D-alanine and, therefore, a less highly cross-linked murein than do hyphae or swarmer cells (Roggentin, 1980; Roggentin and Hirsch, 1982). Fine structural studies of a few isolates have shown intracytoplasmic membrane systems, which develop in older cells under certain growth conditions (Conti and Hirsch, 1965). The major cellular fatty acid in hyphomicrobia is straight-chain unsaturated C_{18:1} (Urakami and Komagata, 1987b). Unusually long-chain hydroxy fatty acids with 24 and 26 carbon atoms have been found in some *Hyphomicrobium* strains (Sittig and Hirsch, 1992). All strains investigated so far contain ubiquinone Q-9 and a small amount of squalene, whereas Hopan-22(29)-ene and Hopan-22(29)-ol are present in only some strains (Urakami and Komagata, 1986b). 2-*o*-methyl-D-mannose has been reported to occur as a component of the extracellular acidic polysaccharide hyphomicran in *Hyphomicrobium* sp. JTS-811. Hyphomicran consists of D-glucose, D-mannose, 2-*o*-methyl-D-mannose, and pyruvic acid residues in relative proportions of 2:1:1:1 (Kanamaru et al., 1982a, b).

Colonial or cultural characteristics Liquid media may either become turbid or remain clear upon growth of hyphomicrobia, depending on the strain. In the latter case, growth occurs as a surface pellicle or ring on the glass walls near the medium sur-

face. Attachment to glass walls may be inhibited by light in some strains (Hirsch and Conti, 1964b). Surface pellicles, when shaken, fall to the bottom of the vessel, especially in older cultures. Colonies on solid media remain quite small, even after long incubation. Some pellicle-forming strains have colonies of variable sizes.

The life cycle of *Hyphomicrobium* species is complicated. It has been studied with special emphasis on the budding process (Kingma-Boltjes, 1936; Mevius, 1953) and on bud nucleation and the possibility of obtaining synchronous swarmer cells (Moore and Hirsch, 1973a; Matzen and Hirsch, 1982a). Up to 12 consecutive buds are formed on one hyphal tip, and the size of swarmer cells produced increases with mother-cell age. Synchronous swarmer cells of nearly identical size have been produced by Matzen and Hirsch (1982a) from chemostat cultures transferred to a glass wool-packed column and washed with fresh medium for several hours. Silver ions (5 ng/ml) increase the time required for growth initiation (Giangiordano and Klein, 1994). Motile swarmer cells of *Hyphomicrobium* sp. W1-1B display a positive chemotactic response toward methylamine, dimethylamine, and trimethylamine, but not toward methanol or arginine (Tuhela et al., 1998).

Metabolism and metabolic pathways All species described so far are methylotrophs; the highest growth yields are obtained with methanol or methylamine as the carbon and energy source. Most strains are also stimulated by a variety of other carbon

sources; however, growth rates and yields are usually substantially lower than with the C_1 compounds (Matzen and Hirsch, 1982b). The uptake of methylamine occurs via a single inducible transport system, irrespective of whether methylamine is used as the carbon and energy source or only as the nitrogen source. Cells grown on other carbon and nitrogen sources do not possess this transport system, but it can be induced by methylamine within 45–50 min. Methylamine uptake is inhibited by azide, cyanide, *N*-ethylmaleimide, and carbonyl cyanide-*m*-chlorophenyl-hydrazone (Brooke and Attwood, 1984). Specialized *Hyphomicrobium* strains can use a variety of other specific C_1 -compounds (Table BXII.α.158).

Hyphomicrobia do not possess an active pyruvate dehydrogenase complex. This makes it impossible to convert pyruvate into acetyl-CoA and to generate energy from carbon compounds containing three or more carbon atoms (Harder et al., 1975).

Some strains are able to grow with C_2 -compounds, such as ethanol and acetate, or with the C_4 -compound β -hydroxybutyrate. In these strains, the activities of the following enzymes have been measured in cell-free extracts: ethanol dehydrogenase, acetaldehyde dehydrogenase, acetothiolase, 3-hydroxybutyrate dehydrogenase, and β -ketothiolase. It has been suggested that when cells are grown on ethanol, acetate, or 3-hydroxybutyrate, acetyl-CoA and energy are formed and reducing power is generated mainly by the tricarboxylic acid cycle, while carbon was assimilated via the glyoxylate cycle, with phosphoenolpyruvate carboxykinase functioning as the main gluconeogenic enzyme (Attwood and Harder, 1974). A mutant lacking methanol dehydrogenase activity has been grown in a medium containing both methylamine and ethanol and does not show diauxic growth. When the concentration of methylamine in the medium falls to 9 ± 0.8 mM, ethanol is utilized and the two substrates are then metabolized simultaneously until the supply of methylamine is exhausted (Brooke and Attwood, 1983).

Methanol is oxidized by a periplasmic methanol dehydrogenase, which contains pyrroloquinoline-quinone (PQQ) as the redox cofactor (Duine and Frank, 1980a) and transfers the electrons to cytochrome c_1 (Dijkstra et al., 1989). Methylamine is metabolized via *N*-methyl-glutamate using an *N*-methyl-glutamate dehydrogenase (Loginova et al., 1976; Meiberg and Harder, 1978). Di- and trimethylamine dehydrogenases are located in the cytoplasm (Kasprzak and Steenkamp, 1983, 1984). Hyphomicrobia contain tetrahydrofolate (H_4F)-linked, as well as tetrahydro-methanopterin (H_4MPT)-linked, C_1 -metabolites. Some interconverting enzymes have been determined (Marison and Attwood, 1982; Vorholt et al., 1999). Molecular analysis has indicated that the chloromethane utilization pathway in *H. chloromethanicum* is similar to the corrinoid-dependent methyl transfer system in *Methylobacterium chloromethanicum* (McAnulla et al., 2001).

The roles of some other NADP-dependent (plus unknown factor) or dye-linked (form)-aldehyde dehydrogenases, some of them with high activity and specificity, remain to be elucidated (Marison and Attwood, 1980; Köhler and Schwartz, 1982; Köhler et al., 1985; Poels and Duine, 1989; Klein et al., 1994; Kessler and Schwartz, 1995).

The dichloromethane-utilizing *Hyphomicrobium* sp. DM2 possesses a type A dichloromethane dehalogenase (Schmid-Appert et al., 1997).

Hyphomicrobia use the serine pathway for carbon assimilation from reduced C_1 -compounds. However, there has been controversy concerning the occurrence of isocitrate lyase in the genus *Hyphomicrobium*. Specific strains possess the icl^+ variant (Bellion

and Spain, 1976; Uebayasi et al., 1985; Yoshida et al., 1995a; Doronina et al., 1996b; Tanaka et al., 1997a). On the other hand, Attwood and Harder (1977), Doronina (1985), and Doronina et al. (1996b) have had evidence for the icl^- variant of the serine pathway in certain strains. Recent observations have confirmed the distribution of unstable isocitrate lyase activities in the genus *Hyphomicrobium*, and the general operation of the icl^+ serine pathway has been suggested (Izumi et al., 1996).

Enzymes for the assimilatory pathways of either C_1^- or C_2^- compounds are regulated coordinately, but separately from the dissimilatory pathway enzymes associated with these compounds (Brooke and Attwood, 1985).

When the nitrogen source is ammonium sulfate or methylamine and the supply is in excess, NADPH-dependent glutamate dehydrogenase is used for the assimilation of nitrogen. In contrast, with a limited nitrogen supply, the cells express high levels of glutamine synthetase and NADH-dependent glutamine:2-oxoglutamate aminotransferase activity, while the activity of glutamate dehydrogenase is lower. When nitrate is the nitrogen source, the glutamine synthetase/glutamine oxoglutamate aminotransferase pathway is used, irrespective of the nitrogen concentration (Brooke et al., 1987). Some strains are able to use allantoin as a nitrogen source (van der Drift et al., 1981). The activity of NH_4^+ -assimilating enzymes is regulated by the C/N ratio in the growth medium (Gräzer-Lampart et al., 1986; Duchars and Attwood, 1989).

Nitrate is reduced anaerobically by some strains; for these organisms, a special enrichment technique using KNO_3 and methanol has been described by Attwood and Harder (1972) and Sperl and Hoare (1971). A fundamental study of the *Hyphomicrobium* denitrifying capacity shows (Timmermans and van Haute, 1983) that these bacteria grow well anaerobically and with identical rates in the presence of methanol and either NO_3^- or NO_2^- . However, denitrification enzymes are present only to a limited extent (Sperl and Hoare, 1971; Vedenina et al., 1991; Kloos et al., 1995; Fesefeldt et al., 1998a, b) or are not expressed (Lebedinskii and Vedenina, 1981). Some strains have the ability to grow with methanol and nitrous oxide as the terminal electron acceptor (Lebedinskii, 1981). *Hyphomicrobium* sp. X can grow anaerobically on di- or trimethylamine in the presence of nitrate (Meiberg and Harder, 1978; Meiberg et al., 1980). *Hyphomicrobium* sp. DM2 has recently been shown to grow with dichloromethane in the absence of oxygen, using nitrate as a terminal electron acceptor (Kohler-Staub et al., 1995). White et al., (1987) have described a *Hyphomicrobium* strain that grows anaerobically on methylsulfate as a carbon and energy source at the expense of nitrate. Electron transport from methanol to nitrate has been described by Lebedinskii and Vedenina (1987).

Cells of *Hyphomicrobium* spp. VS and EG oxidize sulfide to thiosulfate very actively (Suylen et al., 1986; Pol et al., 1994). Yield data and ATP synthesis indicate that further oxidation of thiosulfate is possible (Suylen et al., 1986; Vedenina and Sorokin, 1992). *Hyphomicrobium* sp. 53-49 shows autotrophic growth with $H_2/CO_2/O_2$ and NH_4^+ (Uebayasi et al., 1981, 1984, 1985). Metabolism of monomethyl sulfate remains uncertain. ^{13}C -NMR data are consistent with the hydroxylation of monomethyl sulfate via a monooxygenation mechanism and subsequent spontaneous hydrolysis of the methanediol monosulfate intermediate (Higgins et al., 1996).

Vitamin B_{12} stimulates growth of some species, especially their swarmer cells (Matzen and Hirsch, 1982b), but there is no absolute vitamin requirement in batch cultures. Most strains grow

TABLE BXII.α.158. Growth substrates used by specific undescribed *Hyphomicrobium* strains as sole carbon and energy source

Substrate	<i>Hyphomicrobium</i> species or strain
Acetate, 0.2% (w/w) ^a	MS 72, MS 75; MS 219, MS 223, MS 246, TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Ethanol, 0.5% (w/v) ^a	MS 72, MS 75, MS 219, MS 223, MS 246, TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Ethylamine, 0.5% (w/v) ^b	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Ethylmethylaniline, 0.5% (w/v) ^c	MS 72, MS 75, MS 219, MS 223, MS 246
N-formylglycineethyl ester, 0.3% (w/v) ^d	MS 72, MS 75, MS 219, MS 223, MS 246
N,N-dimethylformamide, 2.5% (w/v) ^e	TNBP221
Chloromethane, 0.1 mM ^{f, g}	CM1, CM2, CM9, CM29, CM35, MCI
Dichloromethane, 10 mM ^h	GJ21, DM2
Sodium monomethylsulfate, 0.5% (w/v) ⁱ	MS 72, MS 75; MS 219, MS 223, MS 246; <i>Hyphomicrobium</i> sp.
Methanesulfonate, 10 mM ^j	P2
Methanethiol ^{k, l}	I55, VS
Dimethylsulfone, 10 mM ^m	S1
Dimethylsulfoxide, 2–4 mM ⁿ	S1, I55, S, VS, EG
Dimethylsulfide, 0.5–1 mM ⁿ	S1, I55, VS, EG
Dimethyldisulfide, 1 mM ^o	VS, MS3
Dimethyltrisulfide, 1 mM ^p	VS
Diethylsulfone, 10 mM ^m	S1
Propanesulfonate, 10 mM ^m	S1
Butanesulfonate, 10 mM ^m	S1
Hexanesulfonate, 10 mM ^m	S1
Dimethylphosphate, 0.5% (w/v) ^b	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Monomethylphosphate, 0.3% (w/v) ^b	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Dimethylphosphonate, 0.3% (w/v) ^{b, q}	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Diethylphosphate, 0.5% (w/v) ^b	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Trimethylphosphate, 0.3% (w/v) ^b	TMPO 1/8, TMPO 1/9, TMPO 1/10, DMMP 1/6, DMMP 1/9, DMMP 1/10, DMMP 1/11, DMMP 1/15, TMPO 2/3, TMPO 2/6, TMPO 2/9, TMPO 2/10, TMPO 2/13, DMPO/HW1-5
Dimethylphosphite, 0.05% (w/v) ^c	MS 72, MS 75, MS 219, MS 223, MS 246
Trimethylphosphite, 0.05% (w/v) ^c	MS 72, MS 75, MS 219, MS 223, MS 246

^aGhisalba and Kuenzi (1983); Ghisalba et al. (1987).^bGhisalba et al. (1987).^cGhisalba and Kuenzi (1983).^dGhisalba et al. (1985).^eShuttleworth (1996).^fDoronina et al. (1996b).^gHartmans et al. (1986).^hDiks et al. (1994a, b); Stucki et al. (1981).ⁱGhisalba and Kuenzi (1983); White et al. (1987).^jDe Marco et al. (2000).^kConcentration data not available.^lCho et al. (1992); Pol et al. (1994).^mBorodina et al. (2000).ⁿBorodina et al. (2000); Cho et al. (1992); De Bont et al. (1981b); Pol et al. (1994); Suylen and Kuenen (1986).^oPol et al. (1994); Smet et al. (1996).^pPol et al. (1994).^qUsed with very low growth rates.

in the presence of 2.5% NaCl, but they also develop at low salt concentrations, approaching that of distilled water. Milk is coagulated by one strain. H₂S evolution and gelatin liquefaction have also been observed in this organism. Some isolates of *Hyphomicrobium* grow at 4–6°C; others can multiply at 45°C (Hirsch, unpublished observations).

Genetics and bacteriophages Genome sizes of three species ranged from 2.13–2.62 × 10⁹ Da (Moore and Hirsch, 1973b; Kölbel-Boelke et al., 1985).

In addition to amino acid auxotrophs, mutants have been found that are specifically defective in methanol oxidation, resistant against antibiotics, or nonmotile and/or morphologically altered (Wieczorek and Hirsch, 1979; Marison and Attwood, 1982; Gliesche and Eckhardt, 1991; Gliesche and Hirsch, 1992). Transposon mutagenesis has been established for *H. facile* IFAM B-522 using transposon Tn5 and its derivatives, Tn7, Tn10, and Tn501 (Gliesche and Hirsch, 1992).

Broad host range IncP-1 plasmids (RP1, RP4, RP4::Muets, R68, R68.45, pMO60, pLUB21, pLUB113) have been successfully

transferred by interspecific matings to *H. facile* IFAM B-522 and *H. denitrificans* DSM 1869^T (Dijkhuizen et al., 1984; Gliesche and Hirsch, 1992). Mermod et al. (1986) have reported the transfer of plasmid pNM185 (a pKT231 derivative) into *H. denitrificans* NCIB11706.

Chromosome mobilization has been demonstrated with the conjugative IncP-1 plasmids RP1, R68.45, and pMO60 into *H. facile* (Gliesche and Hirsch, 1992). Using the IncP helper pRK2013 plasmid, pLA2917 has been introduced into *Hyphomicrobium* species by triparental matings. However, pLA2917 is always found integrated into the chromosome (Gliesche, 1997). Genes *aceE*, *aceF*, and *lpd* from *Escherichia coli* K12 JC6310, which code for the pyruvate dehydrogenase complex, have been expressed in *H. denitrificans* DSM 1869^T, with RP4' *pdh1* resulting in growth and denitrification with pyruvate as the sole carbon source (Dijkhuizen et al., 1984).

An efficient system for electroporation of *H. facile* IFAM H-526^T and *H. denitrificans* DSM 1869^T has been developed with vectors based on the broad-host-range plasmid pBBR1 (Gliesche, 1997).

The occurrence of genes coding for denitrification and nitrogen fixation enzymes has been shown by Southern or dot blot hybridization with gene probes specific for nitrate reductase (*narG*), cytochrome *c*, *d*₁-containing nitrite reductase (*nirS*), Cu-containing nitrite reductase (*nirK*), nitrous oxide reductase (*nosZ*), and nitrogenase reductase (*nifH*). In hyphomicrobia, the Cu-containing nitrite reductase appears to be more common than the cytochrome *c*, *d*₁-containing nitrite reductase (Suzuki et al., 1993; Kloos et al., 1995; Tuhela et al., 1997; Fesefeldt et al., 1998a, b). Southern blot hybridizations have indicated the presence of genes coding for methanesulfonic acid monooxygenase (*msn*) in a methanesulfonate-degrading *Hyphomicrobium* strain (De Marco et al., 2000).

DNA sequence data exist for genes coding for the 5S rDNA (Stackebrandt et al., 1988a; Boulygina et al., 1993), 16S rDNA (Stackebrandt et al., 1988a; Tsuji et al., 1990; Tuhela et al., 1997; Rainey et al., 1998; Borodina et al., 2000; De Marco et al., 2000; Layton et al., 2000; McDonald et al., 2001; Stein et al., 2001), methanol dehydrogenase (*mxhF*; Fesefeldt and Gliesche, 1997; McDonald and Murrell, 1997a; Tanaka et al., 1997b), dimethylamine dehydrogenase (*dmd*; Yang et al., 1995a), methyltransferase gene cluster (*cmu*; McAnulla et al., 2001), Cu-containing nitrite reductase (*nirK*; Braker et al., 1998), isocitrate lyase (Tanaka et al., 1997a), serine-glyoxylate aminotransferase (Hagishita et al., 1996a), serine hydroxymethyltransferase (Miyata et al., 1993), hydroxypyruvate reductase (Yoshida et al., 1994), dichloromethane dehalogenase (*dcmA*; Vuilleumier et al., 1997), methenyl tetrahydromethanopterin cyclohydrolase (*mch*; Vorholt et al., 1999), and unknown gene fragments involved in methanol oxidation (Fesefeldt et al., 1997; Gliesche et al., 1997). Specific PCR primer systems have been described for the amplification of these genes from pure cultures or environmental samples (Miyata et al., 1993; Yoshida et al., 1994; Yang et al., 1995a; Hagishita et al., 1996a; Fesefeldt and Gliesche, 1997; McDonald and Murrell, 1997a; Tanaka et al., 1997a; Vuilleumier et al., 1997; Braker et al., 1998; Vorholt et al., 1999; McAnulla et al., 2001). An identification system for environmental *Hyphomicrobium* isolates, based on denaturing gradient gel electrophoresis of a fragment of the *mxhF* gene, has been described by Fesefeldt and Gliesche (1997).

Partial amino acid sequences exist for the enzymes dimethylamine dehydrogenase and trimethylamine dehydrogenase (Kas-

przak et al., 1983). In *Hyphomicrobium* sp. DM2 and GJ21, the *dcm* region (dichloromethane utilization genes) is associated with the insertion sequences IS1354 (only in DM2), IS1355, and IS1357. Furthermore, multiple copies of these insertion sequences have been found outside the *dcm* region. The high degree of sequence conservation observed within the genomic region responsible for dichloromethane utilization in other aerobic methylophilic bacteria and the occurrence of clusters of insertion sequences in the vicinity of the *dcm* genes suggest that a transposon is involved in the horizontal transfer of these genes among methylophilic bacteria (Schmid-Appert et al., 1997).

Ribosomal RNA cistron homologies among *Hyphomicrobium* strains have been investigated by Moore (1977). Low molecular weight RNA profiles have been used for genotypic identification of several *Hyphomicrobium* species (Höfle, 1990). The 16S–23S rRNA internal transcribed spacer region has been investigated by Scheinert et al. (1996). Poly(A) sequences at the 3'-terminus have been observed in the RNA of *H. facile* (Schultz et al., 1978).

Lytic bacteriophages have been isolated for several strains of *Hyphomicrobium* (Voelz et al., 1971; Kaplan et al., 1976; Yelton et al., 1979; Gliesche et al., 1988; Preissner et al., 1988). The presence of temperate bacteriophages and bacteriocins in *Hyphomicrobium* strains has also been demonstrated (Gliesche et al., 1988; Holm, 1991). *Hyphomicrobium facile* IFAM B-522 (RP4) is not sensitive toward the donor-specific (IncP) phages PRD1 and GU5 (Gliesche and Hirsch, 1992).

Serological relationships among some hyphomicrobial isolates have been studied by Powell et al. (1980).

Pathogenicity Twelve strains have been tested for pathogenicity against mice or guinea pigs; all are avirulent (Famureva et al., 1983).

Ecology and use in biotechnology *Hyphomicrobium* species could be isolated from all soil samples tested so far; they are present in nearly all water samples as well (Hirsch and Conti, 1964a; Hirsch and Rheinheimer, 1968). In freshwater habitats, they are especially prevalent in the neuston layer, on submerged surfaces, and in the upper sediment layer, even under anaerobic conditions. Hyphomicrobia also come from temporary puddles, sewage treatment plants, and the surface of indoor flower-pots (Hirsch, 1974a).

An association of hyphomicrobia with methanotrophs is capable of denitrifying with methanol; growth is possible under high CH₄ and low O₂ conditions (Wilkinson et al., 1974; Amaral et al., 1995). On the other hand, co-immobilized mixtures of *Hyphomicrobium* sp. and methanogenic bacteria are very efficient in simultaneous denitrification and methanogenesis (Lin and Chen, 1995; Zellner et al., 1995).

Using the most-probable-number technique with methanol as the sole carbon source, hyphomicrobia have been enumerated as 0.2% (= 10⁶ g⁻¹ dry weight hyphomicrobia) of the total bacteria determined by acridine-orange direct counts in a clay loam soil (Aa and Olsen, 1996). With the same method, Fesefeldt et al. (1997) have found 2 × 10⁴ g⁻¹ dry weight hyphomicrobia in a garden soil (0.7% of colony-forming units of methylophilic bacteria). The *H. facile* population amounted to 30% of total hyphomicrobia in this soil.

The existence of denitrifying hyphomicrobia is of special interest because of the necessity to remove nitrate at drinking water treatment plants (Liessens et al., 1993) and sewage treatment plants (Schmider and Ottow, 1986; Nyberg et al., 1992; Lee and Welander, 1996; Lemmer et al., 1997). Coenoses consisting of a

Hyphomicrobium sp. and a *Paracoccus* sp. are quite efficient in the removal of both methanol and nitrate (Claus and Kutzner, 1985; Vedenina and Govorukhina, 1988; Neef et al., 1996). Investigation of the community structure in sewage treatment plants has revealed a very high abundance and diversity of hyphomicrobia (Holm et al., 1996). A domestic wastewater treatment plant has been found to contain up to 2×10^4 hyphomicrobia ml⁻¹ in the influent, 9×10^4 – 6×10^5 in activated sludge, 1 – 4×10^3 in the effluent, and 2–12 hyphomicrobia ml⁻¹ in the receiving lake. DNA–DNA hybridizations have classified the isolates into 30 groups (Holm et al., 1996), which can be assigned to Clusters I to IV based on 16S rDNA sequences (Rainey and Stackebrandt, unpublished data). The population of denitrifying *Hyphomicrobium* DNA–DNA-hybridization group HG 27 amounts to approximately 30% of the total facultatively anaerobic hyphomicrobia found in this activated sludge (Gliesche and Fesefeldt, 1998). When Layton et al. (2000) extracted DNA from an industrial wastewater from a treatment plant, they found 16S rDNA sequences from representatives of *Hyphomicrobium* clusters I and II. *Hyphomicrobium* 16S rRNA comprised approximately 5% of the 16S rRNA in the activated sludge of this treatment plant.

16S rDNA genes related to *Hyphomicrobium* spp. have been amplified from total community DNA extracted from a water sample of the northern portion of Green Bay (40 m depth) (Stein et al., 2001).

Hyphomicrobia have also been used for the removal of odorous, volatile sulfur compounds, such as hydrogen sulfide, methanethiol, dimethylsulfide (Zhang et al., 1991; Cho et al., 1992; Pol et al., 1994; Smet et al., 1996), and dichloromethane (Diks et al., 1994a, b) from air and gases. Specific *Hyphomicrobium* strains and enzymes have been applied in biosensors for the detection of methanol (Argall and Smith, 1993), methylsulfates (Schär and Ghisalbal, 1985; Ghisalbal et al., 1986), trimethylamine (Large and McDougal, 1975; Wong and Gill, 1987), dihalomethanes (Gälli and Leisinger 1985; Henrysson and Mattiasson, 1993), L-serine, and glyoxylate (Yoshida et al., 1993). A coenosis consisting of *Hyphomicrobium* sp. DM2 and *Rhodococcus rhodochrous* OFS has efficiently degraded dichloromethane, 2-propanol, and methanol in a gas lift loop bioreactor (Vanderberg-Twary et al., 1997).

Hyphomicrobium methylovorum has been used for the production of L-serine (Yamada et al., 1986; Izumi et al., 1993).

Other studies *Hyphomicrobium* species have been investigated for fatty acids (Auran and Schmidt, 1972; Ikemoto et al., 1978a; Eckhardt et al., 1979; Urakami and Komagata, 1979, 1987a, b; Vedenina et al., 1991; Sittig and Hirsch, 1992), phospholipids (Goldfine and Hagen, 1968; Guckert et al., 1991; Sittig and Hirsch, 1992; Batrakov and Nikitin, 1996), hopanoids (Rohmer et al., 1984), ubiquinones (Köhler and Schwartz, 1981; Urakami and Komagata, 1981, 1986b; Sittig and Hirsch, 1992), cytochromes (Large et al., 1979; Köhler and Schwartz, 1983; Dijkstra et al., 1988a, b, 1989; Frank and Duine, 1990a), pyrroloquinoline quinone (Duine et al., 1980, 1981, 1990; De Beer et al., 1983; Houck et al., 1989), and poly-β-hydroxybutyrate (Jacobsen, 1975). The following enzymes have been purified and characterized in more detail: methanol dehydrogenase (Duine et al., 1978; Duine and Frank, 1979, 1980a, b; Schär et al., 1985; Miyazaki et al., 1987a; Frank et al., 1988; Frank and Duine, 1990b; Geerloff et al., 1994b), dimethylamine dehydrogenase (Kasprzak and Steenkamp, 1984; Meiberg and Harder, 1979; Steenkamp 1979; Steenkamp and Beinert, 1982a, b), trimethylamine dehydrogenase (Steenkamp 1979; Steenkamp and Beinert, 1982a, b;

Kasprzak et al., 1983), dye-linked aldehyde dehydrogenase (Marison and Attwood, 1980; Köhler and Schwartz, 1982), dye-linked formaldehyde dehydrogenase (Klein et al., 1994), NAD-linked, GSH- and factor-independent aldehyde dehydrogenase (Poels and Duine, 1989; Duine, 1990), NADP⁺-dependent glutamate dehydrogenase (Duchars and Attwood, 1987), glutamine synthetase (Duchars and Attwood, 1991), hydroxypyruvate reductase (Goldberg et al., 1992, 1994; Yoshida et al., 1994; Hagishita et al., 1996b; Izumi et al., 1996), serine–glyoxylate aminotransferase (Hagishita et al., 1996a, b; Izumi et al., 1996), glycerate kinase (Hill and Attwood, 1974; Yoshida et al., 1992; Izumi et al., 1996), phosphoenolpyruvate carboxylase (Yoshida et al., 1995b; Izumi et al., 1996), methyl mercaptan oxidase (Suylen et al., 1987), dichloromethane dehalogenase (Köhler-Staub and Leisinger, 1985; Köhler-Staub et al., 1986; Leisinger and Köhler-Staub, 1990), dimethyl sulfoxide reductase (Hatton et al., 1994), phosphoglycerate mutase (Hill and Attwood, 1976a, b), serine hydroxymethyltransferase (Miyazaki et al., 1986, 1987b, c, d), and copper-containing nitrite reductase (Suzuki et al., 1993).

ENRICHMENT AND ISOLATION PROCEDURES

A variety of enrichment techniques has been proposed. In all cases, growth of hyphomicrobia is slow; they may be overgrown in the presence of other heterotrophic bacteria. Most enrichment cultures for nitrifying bacteria contain hyphomicrobia in large numbers (Stutzer and Hartleb, 1988; Hirsch and Rheinheimer, 1968; Hirsch, 1970). Under oligotrophic conditions and after prolonged incubation, hyphomicrobia usually outcompete other bacteria. A slow, but successful method consists of keeping a natural water sample at room temperature in the dark for several weeks or months. Eventually, hyphomicrobia become part of the dominant microflora, even in the presence of amoebae. Another method prescribes the addition to a natural water sample or soil suspension of methylamine hydrochloride at 3.38 g/l and/or incubation in an atmosphere of methanol (Hirsch, 1970). Such an enrichment should be monitored frequently in order to determine the optimal time for subculturing. Inoculation of a natural sample into mineral salts medium 337, containing 0.1–0.2% (w/v) of a C₁-compound, and dark incubation at 20–25°C for a few weeks usually yields hyphomicrobia in large numbers (Hirsch and Conti, 1965). Improvements of medium 337¹ that result in faster growth and higher yields have been reported by Matzen and Hirsch (1982b). Several modifications of medium 337-B (Matzen and Hirsch, 1982b) have been developed for specific genetic and physiological applications and for the isolation of a greater *Hyphomicrobium* diversity from natural habitats (Table BXII.α.159).

Isolation of rosette-forming and denitrifying hyphomicrobia from aquatic sediments has been achieved by inoculating sediment samples into a mineral salts medium containing 5 g/l KNO₃ and up to 0.5% (v/v) of methanol; incubation is anaerobic at room temperature (Attwood and Harder, 1972). It must be stressed, however, that all of these methods will yield different *Hyphomicrobium* species. The application of an oligotrophic medium containing low concentrations of peptone, yeast extract, and glucose ("PYG"; Staley, 1968) has often yielded morphologically *Hyphomicrobium*-like bacteria. Such isolates usually do not

1. Composition of medium 337: KH₂PO₄, 1.36 g; Na₂HPO₄, 2.13 g; MgSO₄·7H₂O, 0.3 g; (NH₄)₂SO₄, 0.5 g; CaCl₂·2H₂O, 1.99 mg; FeSO₄·7H₂O, 1.0 mg; MnSO₄·H₂O, 0.35 mg; Na₂MoO₄·2H₂O, 0.5 mg; vitamin B₁₂ (if needed), 2.5 µg; distilled water, to 1000 ml; pH, 7.2.

TABLE BXII.α.159. Growth media for specific applications with hyphomicrobia

Medium	Characteristics and composition	Application
337 ^a	Mineral salts medium (carbon source: methylamine or methanol)	Enrichment, isolation, and cultivation of hyphomicrobia
337a ^b	Modification of medium 337 with 3.38 g/l methylamine and reduction of the trace elements	Enrichment, isolation, and cultivation of hyphomicrobia
337-MA ^c	337a with 3.38 g/l methylamine	Enrichment, isolation, and cultivation of hyphomicrobia
337-M ^c	337a with 0.5% (v/v) methanol	Enrichment, isolation, and cultivation of hyphomicrobia
337-B ^c	337a with 2.5 g/l vitamin B ₁₂ , 0.3 g/l L-lysine and 5.0 g/l Na-gluconate (C-source: 3.38 g/l methylamine-HCl or 0.5% (v/v) methanol)	Optimized medium for <i>Hyphomicrobium facile</i>
337-B1 ^d	337-B with 3.38/l methylamine-HCl, but without vitamin B ₁₂ , L-lysine, or gluconate	Standard minimal medium for the cultivation of all hyphomicrobia growing on methylamine
337-B2 ^d	337-B1 with 0.2% (w/v) Bacto nutrient broth and 0.2% (w/v) Casamino acids	Isolation of auxotrophic mutants
337-B3 ^d	337-B1 and 0.075% (w/v) Bacto nutrient broth and 0.075% (w/v) Casamino acids	Interspecific matings
337-B4 ^e	337-B1 and 2.5 g/l vitamin B ₁₂	Cultivation of vitamin B ₁₂ -dependent hyphomicrobia
337-B5 ^e	337-B4 and 0.25 g/l peptone, 20 ml/l HBM and 10 ml/l vitamin solution No. 6 ^f	Enrichment, isolation, and cultivation of specific hyphomicrobia from sewage
337-B6 ^g	337-B5 and 0.075% (w/v) Bacto nutrient broth and 0.075% (w/v) Casamino acids	Interspecific matings with hyphomicrobia with optimal growth on medium 337-B5
337-B7 ^h	337-B5 with 5 mM NaNO ₃ and 0.5% (v/v) methanol instead of methylamine	Determination of the denitrification activity
337-B8 ⁱ	337-B1 with 0.5% (v/v) methanol instead of methylamine	Standard minimal medium for the cultivation of all hyphomicrobia growing on methanol
337-B9 ^j	337-B8 with 0.5% (w/v) KNO ₃	Enrichment, isolation, and cultivation of denitrifying hyphomicrobia
337-B10 ^k	337-B5 with 0.5% (v/v) methanol instead of methylamine-HCl	Cultivation of specific hyphomicrobia from sewage
337-B11 ^k	337-B5 with 5 mM KNO ₃ and 0.5% (v/v) methanol instead of methylamine-HCl	Cultivation of denitrifying hyphomicrobia with optimal growth on medium 337-B5
DST ^l	Mineral salts, trace elements, 1% (w/v) Difco agar, and an atmosphere of 1% (v/v) CH ₃ Cl	Enrichment and cultivation of hyphomicrobia that utilize chloromethane

^aData from Hirsch and Conti (1964b).^bData from Moore and Hirsch (1973a).^cData from Matzen and Hirsch (1982b).^dData from Gliesche and Hirsch (1992).^eData from Holm et al. (1996).^fHBM and Vitamin Solution No. 6 according to Schlesner (1994).^gData from Gliesche et al. (1997).^hData from Fesefeldt et al. (1998b).ⁱData from Gliesche and Fesefeldt (1998).^jData from Gliesche and Fesefeldt (1998).^kData from Fesefeldt (1998).^lData from Doronina et al. (1996b).

grow on C₁ compounds and are often pigmented; they comprise a different group of generic rank (see *Hirschia* spp.; Schlesner et al., 1990).

Hyphomicrobia growing on chloromethane can be enriched for with the addition of 1% (w/v) CH₃Cl to a mineral salts medium. Subsequently, this can be plated on the same medium solidified with 1% Difco agar and containing 0.01% (w/v) bromothymol blue to indicate the release of HCl (Doronina et al., 1996b).

Once hyphomicrobia occur or predominate in liquid enrichments, they can be isolated and purified by repeatedly streaking enrichments on mineral salts medium "337" and incubating the plates in the dark at 20–25°C. To avoid excessive drying of the plates, they should be placed in plastic bags, or thicker layers of agar should be used. Concerning the purification procedure, it should be remembered that pellicle-forming strains (e.g., NQ-521Gr) usually produce colonies of quite different size. Subculturing small or large colonies results again in growth of both types.

Hyphomicrobium colonies are often tough and coherent; before

spreading on plates, they should be ground up properly. Typical colonies of hyphomicrobia appear dark brown in transmitted light, often with folds and concentric rings. Under reflected light, the colonies of many strains are shiny and bright beige or even colorless.

MAINTENANCE PROCEDURES

Most hyphomicrobia can be kept well at 4–5°C when growing on slants. Subculturing every 5–6 months is sufficient. Lyophilization in skim milk is the optimal method for maintenance of most cultures. Suspension in phosphate buffer and sterile glycerol, followed by immediate vortexing and cooling down to –25°C, is another technique; such preparations may be kept for several years in the freezing compartment of a refrigerator. For subculturing, the glycerol suspension is streaked directly onto agar plates. Warming up of the glycerol suspension should be avoided, since rapid death of the cells results. Doronina and Trotsenko (1992) have successfully stored hyphomicrobia that grew with chloromethane by deep-freezing these on Whatman paper without cryoprotectants at –40°C to –80°C.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Cell shape and morphogenesis An agar slide culture is prepared with medium "337" and 1.8% Bacto Noble agar (Hirsch, unpublished). The sterile agar medium is spread thinly over sterile glass slides and allowed to solidify. Using sterile coverslips, agar is then cut off to leave two agar squares side-by-side and separated only by a small ditch. One of the squares is inoculated with a thin *Hyphomicrobium* suspension; the other square receives a small droplet of 0.5% methanol. Both squares are covered together with one large coverslip, and the edges can be sealed with a Vaseline-paraffin mixture. Spreading of methanol to the inoculated square should be avoided, since direct contact at this concentration is toxic for the bacteria. Growth and morphogenesis can then be followed over a period of up to 48 h or more.

Cell size measurements Only living cells, preferentially those in agar slide cultures, are used for size measurements. Phase-contrast light micrographs are prepared and enlarged 10 times; sizes are measured on these enlargements from at least 50–100 cells, since considerable size variations exist in asynchronous *Hyphomicrobium* cultures.

Growth on carbon sources Medium 337 is used as a base, and sterile-filtered carbon sources are added at 0.1–0.2% (w/v). Most hyphomicrobia are oligocarbophilic, i.e., they grow (although slowly) at the expense of contaminants in the laboratory air. It is mandatory, therefore, to have control plates inoculated, which do not contain the carbon source to be tested; growth on these controls has to be considered. Growth on plates can be scored after 1, 2, and 4 weeks. Furthermore, oligotrophic growth makes it necessary to subculture at least two additional times with the same carbon source to ensure that the growth observed is due to the substrate offered and not to substrate carried over with the inoculum. Liquid carbon utilization tests can be scored by measuring optical density at 650 nm (OD_{650}) if cells grow turbidly. However, dramatic changes of cell size and morphology can result from some carbon sources, and light microscopy is required to ascertain that this is not the case. Protein determinations are applied widely as a better method for growth estimation (Matzen and Hirsch, 1982b). In all such experiments, it is crucial to have very homogeneous suspensions in the initial inoculation procedure.

Growth stimulation by vitamins Growth of some hyphomicrobia is markedly stimulated by vitamin B₁₂, especially in chemostat cultures where the population consists mainly of very young cells. Obviously, nutrient requirements can change with cell age. Static asynchronous cultures contain large numbers of older mother cells and do not require B₁₂ addition for growth. It has been found, however, that application of a vitamin mixture (Vitamin Solution no. 6; van Ert and Staley, 1971) leads to less stimulation than does the application of B₁₂ alone (Matzen and Hirsch, 1982b).

Growth inhibition by visible light Hyphomicrobia that form surface pellicles rather than turbidity are markedly inhibited by light (Hirsch and Conti, 1964b). Experiments to determine the influence of light on *Hyphomicrobium* growth can be carried out with sunlight illumination of agar plates or liquid cultures. Care must be taken to ensure temperature constancy and to avoid water condensation and/or drying of the plates. In the case of liquid cultures, the light inhibition can be detected by the failure of swarmer cells to attach to glass walls on the illuminated side.

DNA extraction procedures Lysis of hyphomicrobia is often difficult; a variety of techniques has been described for DNA extraction (Gebers et al., 1985). For the "cell mill A" method, 1–2 g of bacterial wet weight are suspended in 20 ml of saline-EDTA supplemented with 1 mg proteinase K. Then, 50 g glass beads (0.1 mm diameter) are added, and the precooled mixture is shaken for 5–10 s in an MSK cell homogenizer (Braun, Mel-sungen, Germany). Cell lysis is completed by adding 20 mg sodium dodecyl sulfate ml⁻¹ to the suspension. For DNA extraction, NaClO₄ and chloroform-isoamyl alcohol are added, and the suspension is shaken for 15 min at 100 rpm. Centrifugation at 1350 × g for 20 min results in separation of the emulsion into two layers. The nucleic acids can be precipitated from the aqueous phase. This is followed by a 45 min ribonuclease treatment and by a 2 h treatment with proteinase K (200 µg/ml) at 37°C. Then, 1 volume of phenol is added (saturated with 1 × SSC [0.15 M NaCl + 0.015 M Na₃-citrate], pH 7) and 0.1 volume of chloroform-isoamyl alcohol is added and the preparation agitated for 10 min at 100 rpm. Centrifugation at 27,000 × g for 20 min results in the separation of the emulsion into three layers, the upper one of which is used for the precipitation of DNA by ethanol. In some cases, a similar method described by Gebers et al. (1981b) and called the "enzyme A" method yields better results.

Extraction of genomic DNA for PCR

Procedure I of Gliesche et al. (1997) Late-exponential-phase cultures (500 ml; 400–500 mg wet weight) are harvested by centrifugation (8000 × g, 45 min, 4°C), resuspended in 1.0 ml double-distilled water with 4.0 ml acetone (4°C), and incubated for 30 min at 0°C. The pellet (4000 × g, 10 min) is dried and resuspended in 4.0 ml buffer 1 (50 mM EDTA, 30 mM Tris, and 5 mM NaCl). Then, 1.0 ml of a lysozyme solution (20 mg lysozyme ml⁻¹ TE [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]) is added and the mixture incubated for 45 min at 37°C. Cells are lysed by the addition of 1.0 ml SDS (10% w/v) and 130 µl of proteinase K solution (20 mg/ml phosphate buffer [0.1 M K₂HPO₄, 0.1 M KH₂PO₄, pH 6.25]), followed by incubation at 56°C for 3 h. After lysis, 275 µl of 5 M NaCl (final concentration 1%, w/v) are added and the suspension gently mixed and incubated for 1 h at 0°C. The cell debris is then pelleted for 30 min at 24,000 × g. High molecular weight genomic DNA is precipitated from the aqueous phase with 0.7–1 volume of isopropanol (20 min, 24,000 × g, 4°C) and air-dried (37°C, 20 min). The resulting pellet is resuspended in 4.0 ml TE. After gently shaking in a water bath (30°C, overnight), 60 µl of RNase A (10 mg/ml in TE) are added, and the suspension incubated for 45 min at 37°C. The lysate is extracted two to three times (6500 × g, 15 min, 4°C) with phenol (saturated with SSC [0.15 M NaCl + 0.015 M trisodium citrate, pH 7.5]), phenol/chloroform (until the interface is clear), and chloroform/isoamyl alcohol (24:1). DNA is precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5.2; final concentration of 0.3 M), and three volumes of 96% ethanol (0°C, 30 min–4 h). The pellet (6000 × g, 30 min, 4°C) is washed once with 10 ml ethanol (70%), dried briefly in air at room temperature, and resuspended in 300–500 µl TE by gentle shaking.

Procedure II of Fesefeldt and Gliesche (1997) Cells from 200 ml late exponential phase *Hyphomicrobium* cultures are harvested by centrifugation (8000 × g, 45 min, 4°C). 100 mg cells (wet weight) are resuspended in 300 µl of 1 × SSC. This cell suspension,

together with 200 mg glass beads (0.10–0.11 mm diameter; Braun, Melsungen) and 60 μ l of a 10% (v/v) SDS are filled into 2.0 ml microtubes with screw cap closures (Sarstedt, Germany) and shaken at 2000 rpm on a rotary shaker 3300 (Eppendorf, Hamburg) for 5 (IFAM P-645) or 15 min. Then, 1.0 ml phenol (65°C; saturated with SSC) is added. After 2 min of gentle shaking by hand, the cell debris and glass beads are pelleted for 30 min at 14,000 rpm and 4°C (Centrifuge 5415C, Eppendorf). The aqueous phase (300–400 μ l) is transferred to a fresh microtube. Purification and precipitation of nucleic acids are performed as described in procedure I. The nucleic acids are resuspended in 200 μ l TE by gentle shaking. After adding 10 μ l RNase A (50,000 U/ml in TE), the suspension is incubated for 30 min at 37°C. From this, DNA is purified and precipitated as in procedure I and resuspended in 100 μ l TE by gentle shaking.

Isolation of plasmid DNA (Gliesche, 1997) Late exponential phase cultures (200 ml; 100–200 mg wet weight) are harvested by centrifugation (8000 \times g, 45 min, 4°C), resuspended in 0.3 ml double distilled water with 1.5 ml acetone (4°C), and incubated for 15 min at 0°C. The pellet (10,000 \times g, 10 min) is dried with air (5 min), resuspended in 300 μ l buffer A (50 mM EDTA, 30 mM Tris, and 5 mM NaCl) and 45 μ l of a lysozyme solution (20 mg/ml TE), and incubated for 45 min at 37°C. Then 60 μ l of Proteinase K solution (20 mg/ml phosphate buffer [0.1 M K_2HPO_4 , 0.1 M KH_2PO_4 , pH 6.25]) are added, followed by incubation at 55°C for 2 h. Cells are harvested (10,000 \times g, 10 min, 4°C) and resuspended in buffer 1 (QIAGEN, Germany). Plasmid DNA is isolated and purified with the QIAGEN Plasmid Maxi Kit, according to the manufacturer's instructions.

DIFFERENTIATION OF THE GENUS *HYPHOMICROBIUM* FROM OTHER GENERA

All *Hyphomicrobium* species described so far grow with C_1 compounds, especially methanol, although methanol was not mentioned in the initial description of the type species, *H. vulgare*, which grows well on formate (Stutzer and Hartleb, 1898). C_1 compounds do not support growth of any of the other morphologically similar genera listed in Table BXII.α.160. Cell shape, arrangement of the bud on the hyphal tip, and the tapering of prosthecae are the best differential characters for this group of budding bacteria. It should be pointed out, however, that the cell shape can vary with the carbon source, growth temperature, nutrient concentration, etc.

Natural samples may sometimes contain organisms that superficially resemble *Hyphomicrobium* species. There are bacteria, such as *Achromatium oxaliferum* from acid bog lakes, which undergo constrictive division, and the "umbilical cord" which holds the two daughter cells of this species together may be mistaken for a thin hyphae (Hirsch, 1974a). Fell (1966) has described *Sterigmatomyces* species, which are yeasts that have a surprising resemblance to hyphomicrobia, except that the cells are much larger and usually carry true vacuoles, as eucaryotes do.

TAXONOMIC COMMENTS

The genus *Hyphomicrobium*—considered monospecific until recently—has been defined mainly by morphological characters and by its conspicuous life cycle. Consequently, many authors have named their isolates *H. vulgare* because they saw mother cells, hyphae, and motile swarmer cells (Kingma-Boltjes, 1936; Mevius, 1953; Zavarzin, 1960; Hirsch and Conti, 1964a; Shishkina and Trotsenko, 1974; Lebedinskii, 1981). Often, the authors have

noted substantial morphological differences among their isolates (Hirsch and Conti, 1964a; Lebedinskii, 1981), and thus it has become increasingly clear that *H. vulgare* is apparently composed of several different species. A detailed study has been conducted on the taxonomy of some 80 C_1 -utilizing hyphomicrobia (Hirsch, unpublished data). A computer analysis of these strains carried out by R. Colwell has revealed eight different groups, separated by similarity values of 50–65%. This must be taken as an indication of the need to separate these isolates on a higher level than that of species. A confirmation of these results comes from DNA–DNA hybridization data obtained with 19 *Hyphomicrobium* strains (Table BXII.α.161; Gebers et al., 1986). Those strains that fall within the eight computer-selected groups show high homology (86–100%), but the homology between members of different groups is, in most cases, below 10%. The differences among the *Hyphomicrobium* species shown in Tables BXII.α.162, BXII.α.163, and BXII.α.164 concern morphological, physiological, and ecological characteristics.

A large number of *Hyphomicrobium*-like bacteria that form hyphae and buds from mother cells and cannot usually grow with C_1 compounds can be found in culture collections. These require at least low concentrations of peptone, yeast extract, etc. In some cases, such organisms have been called "*Hyphomonas*", but information is still lacking on the properties of such strains. Mol% G + C values of these "organic hyphomicrobia" may be lower than those of the C_1 hyphomicrobia (Gebers et al., 1985).

Hyphomicrobium neptunium Leifson 1964 has been isolated from seawater and does not resemble C_1 -utilizing hyphomicrobia, other than with respect to morphology and life cycle. Recently, this organism has been compared with *Hyphomonas* species (Moore and Hirsch, 1972; Hirsch, 1974a, b; Weiner et al., 1985), and Moore et al. (1984) has subsequently transferred *H. neptunium* to the genus *Hyphomonas*.

A bacterium isolated from seawater by Weisrock and Johnson (1966) and supposedly resembling hyphomicrobia has been described as *Hyphomicrobium indicum*. It has already been pointed out by Hirsch (1974a, b) that there exist substantial differences between this facultatively anaerobic organism and the type species, *Hyphomicrobium vulgare*. *H. indicum* forms acid from sugars, is indole, nitrate, and H_2S positive, and deaminates phenylalanine. It is psychrotrophic, requires 50–100% seawater, and lacks true budding and hyphal branching. The mol% G + C of the DNA from *Hyphomicrobium indicum* is 40, in contrast to all other, true hyphomicrobia, which have a range of 59–65. This bacterium should no longer be included in the genus *Hyphomicrobium*.

A budding bacterium labeled "*Hyphomicrobium variabile*" is available from culture collections; it is a patent strain, and further information on its properties is lacking.

The definition of the genus *Hyphomicrobium* has been hampered seriously by the lack of a type culture; *H. vulgare*, as described by Stutzer and Hartleb (1898), no longer exists. A search for a neotype culture has been difficult, since not all of the tests described in the 1898 publication can be used only partly or checked at the present time. Among the 80 C_1 -utilizing hyphomicrobia mentioned above, strain IFAM MC-750 has been found to have properties essentially identical to those of *H. vulgare*. This strain also shows a high similarity to the hypothetical median organism of the 80 hyphomicrobia (Colwell and Hirsch, unpublished data). It has been proposed, therefore, to accept this strain as the neotype culture for the genus *Hyphomicrobium* (Hirsch, 1989). DNA–DNA hybridization studies of 19 hyphomicrobia indicate at least weak relationships between IFAM MC-750 (Institut

TABLE BXII.α.160. Differentiation of the genus *Hyphomicrobium* from other closely related or morphologically similar genera^a

Characteristic	<i>Hyphomicrobium</i>	<i>Ancalomicrobium</i>	"Bacterium T" ^b	<i>Dichotomicrobium</i> ^c	<i>Filomicrobium</i> ^d	<i>Hirschia</i> ^e	<i>Hyphomonas</i>	<i>Pedomicrobium</i>	<i>Prosthecomicrobium</i>
Cells ovoid, pear-, or bean-shaped	+	—	+	—	—	+	+	+	+
Cells nearly spherical	—	—	—	—	—	—	+	—	—
Cells nearly tetrahedral	—	+	v	+	—	—	—	—	—
Cells fusiform	—	—	—	—	+	—	—	—	—
Hyphae formed regularly	+	—	+	+	+	+	+	+	—
Prosthecae normally tapering	—	+	—	—	—	—	—	—	+
Bud elongates with long axis of hypha	+	—	+	+	+	+	+	—	—
Bud elongates perpendicular to hyphal long axis	—	—	—	—	—	—	—	+	—
C ₁ compounds support growth	+	—	—	—	—	—	—	—	—
Moderately thermophilic	—	—	—	+	—	—	—	—	—
Moderately halophilic or halotolerant	—	—	—	+	+	+	+	—	—
Fe and/or Mn are oxidized	v	—	—	—	—	—	—	+	—
May possess gas vesicles	—	+	—	—	—	—	—	—	+

^aSymbols: +, 90% or more of strains are positive; —, 90% or more of strains are negative; v, variable, depending on growth conditions.

^bData from Eckhardt et al. (1979).

^cData from Hirsch and Hoffmann (1989a).

^dData from Schlesner (1987).

^eData from Schlesner et al. (1990).

für Allgemeine Mikrobiologie, Kiel, Germany) and *H. aestuarii* (18–24% homology), *H. zavarzinii* (14%), *H. hollandicum* (11–12%), and *H. facile* (4–11%) (Gebers et al., 1986; Table BXII.α.161).

Euzéby (1998b) has recommended that *Hyphomicrobium facilis*, *Hyphomicrobium facilis* subsp. *facilis*, *Hyphomicrobium facilis* subsp. *tolerans*, and *Hyphomicrobium facilis* subsp. *ureaphilum* (Hirsch, 1989) be changed to *Hyphomicrobium facile*, *Hyphomicrobium facile* subsp. *facile*, *Hyphomicrobium facile* subsp. *tolerans*, and *Hyphomicrobium facile* subsp. *ureaphilum*, respectively.

ACKNOWLEDGMENTS

Donation of cultures by M. Feil, C. Gliesche, W. Harder, N. Holm, T.Y. Kingma-Boltjes, E. Leifson, M. Macpherson-Kraviec, G.T. Sperl, B. Speralski, Y. Trotsenko, R. Weiner, G.A. Zavarzin, and many others is gratefully acknowledged. Critical discussions with R. Colwell, R. Gebers, W. Harder, T. Roggentin, A. Schwartz, and R. Weiner helped in organizing this material. This work would not have been possible without the able technical

assistance of G. Maisch, A. Graeter, B. Hoffmann, M. Beese, M. Kusche, S. Liedtke, and K. Lutter-Mohr.

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *HYPHOMICROBIUM*

The differential characteristics of the species of *Hyphomicrobium* are given in Table BXII.α.162, morphological characteristics of

the species are listed in Table BXII.α.163, and physiological properties are listed in Table BXII.α.164.

List of species of the genus *Hyphomicrobium*

1. ***Hyphomicrobium vulgare*** Stutzer and Hartleb 1898, 76^{AL}. *vul.ga' re*. L. neut. adj. *vulgare* common.

The following description is based on characteristics given by Stutzer and Hartleb (1898) and those of the neo-type strain, IFAM MC-750. Mother cells are oval, pear-, or drop-shaped, 0.5–1.2 × 1–3 μm, with prosthecae (hyphae) of varying lengths and rather constant diameter (0.2–0.3 μm; up to 0.4 μm when stained). In liquids, growth occurs as turbidity or rarely as pellicle and turbidity; cells do not form rosettes. Swarmer cells have one to three subpolar flagella. Colonies remain small, even after long in-

cubation; they are colorless or beige, and brownish in transmitted light. Colony surface shiny but granular, the edge is wavy.

Chemoorganotrophic, aerobic, oligocarbophilic. Grow well with methanol, methylamine·HCl, formate, acetate, *n*-butyrate, isovalerate, propionate, lactate (except for IFAM MC-750), isobutanol, glycerol, L-arabinose, D-mannose, D-melibiose, raffinose, dextrin, amygdalin, esculin, D-glucosamine, N-acetylglucosamine, dilute human urine, succinate, or asparagine. Growth is slow, but stimulated by pyruvate, α-oxoglutarate, β-hydroxybutyrate, oxalate, galacturonate, chitin, lactose, or D-maltose. Most amino acids are inhibi-

TABLE BXII.α.161. Levels of DNA-DNA homologies of *Hyphomicrobium* species (Gebers et al., 1986)

Characteristic	<i>H. vulgare</i> ^T	<i>H. aestuarii</i> ^T	<i>H. aestuarii</i>	<i>H. aestuarii</i>	<i>H. denitrificans</i> ^T	<i>H. facile</i> subsp. <i>facile</i> ^T	<i>H. facile</i> subsp. <i>tolerans</i> ^T	<i>H. facile</i> subsp. <i>tolerans</i>	<i>H. facile</i> subsp. <i>wreghthilum</i> ^T	<i>H. facile</i>	<i>H. hollandicum</i> ^T	<i>H. hollandicum</i>	<i>H. zavarzini</i> ^T	<i>H. zavarzini</i>
Collection number:	MC-750	NQ-521Gr	EA-617	MEV-533Gr	WH-563	TK 0415T	H-526	I-551	CO-558	CO-582	B-522	F-550	KB-677	MC-651
IFAM	27500	27483	64.11	27488	63.09	60.0-61.0 ^b	27485	27489	27491	27492	27484	27498	27498	27497
ATCC	61.38	64.11	63.46	64.68	63.09	60.0-61.0 ^b	59.53	59.4	59.78	60.54	59.34	59.91	62.41	62.91
Mol% G + C ^a														
% Homology with labeled DNA from:														
MC-750	100	18	20	24	19	10	5	5	7	11	4	5	12	11
NQ-521Gr	4	100	110	103	92	13		1		1	0		3	
EA-617 ^a		101	100	70	102		3				3		10	
B-522	2	5	2	4	4		88	86	91	87	100	106	4	
ZV-622	3	9						1		2	2		4	
TK 0415 ^d	10	13				100							13	

^aData are from *T_m* determinations (Gebers et al., 1985).

^bData are from Urakami et al. (1995b).

^cData are from *Bd* determinations (Mandel et al., 1972).

^dData are from Moore and Hirsch (1972).

TABLE BXII.α.162. Characteristics differentiating the species of the genus *Hyphomicrobium*^{a, b}

Characteristics	<i>H. vulgare</i> Stutzer and Hartleb 1898	<i>H. vulgare</i> IFAM MC-750	<i>H. aestuarii</i>	<i>H. chloromethanicum</i>	<i>H. coagulans</i>	<i>H. denitrificans</i>	<i>H. facile</i> subsp. <i>facile</i>	<i>H. facile</i> subsp. <i>tolerans</i>	<i>H. facile</i> subsp. <i>ureaphilum</i>	<i>H. hollandicum</i>	<i>H. methylovorum</i>	<i>H. zavarzinii</i>
Mother cells bean-shaped	—	—	+	+	—	—	—	—	—	—	—	—
Mother cells oval or pear-shaped	+	+	—	+	+	+	+	+	+	+	+	+
Rosette formation	—	—	—	nd	—	+	—	—	—	—	—	+
Pellicle formed on liquids	v	—	+	nd	+	—	—	—	—	v	—	v
Maximum pH >7.5	—	—	—	—	+	—	—	—	—	—	—	—
Growth with CH ₃ Cl	nd	—	—	+	nd	—	—	—	—	—	nd	—
Growth with peptones	—	—	+	nd	+	—	—	+	—	—	nd	+
Growth with acetate	+	+	+	nd	—	(+)	(+)	—	—	—	—	(+)
Gelatin liquefied, milk coagulated	—	—	—	nd	+	nd	—	—	—	—	—	—
Isolated from soil	+	+	—	+	+	+	+	+	+	—	+	+
DNA mol% G + C (<i>T_m</i>)	nd	61	64	60	nd	60–61	60	59	61	62	61	65

^aSymbols: —, 90% or more of strains are negative; +, 90% or more of strains are positive; v, variable, depending on growth conditions; nd, not determined; (+), growth stimulation weak but significant.

^bStrains: *H. vulgare* Stutzer and Hartleb 1898; *H. vulgare* IFAM MC-750; *H. aestuarii* IFAM NQ-521Gr; *H. chloromethanicum* CM2; *H. coagulans* 10-2; *H. denitrificans* TK 0415; *H. facile* subsp. *facile* IFAM H-526; *H. facile* subsp. *tolerans* IFAM I-551; *H. facile* subsp. *ureaphilum* IFAM CO-582; *H. hollandicum* IFAM KB-677; *H. methylovorum* KM-146; *H. zavarzinii* IFAM ZV-622.

TABLE BXII.α.163. Morphological characteristics of the species of the genus *Hyphomicrobium*^{a, b}

Characteristics	<i>H. vulgare</i> Stutzer and Hartleb 1898	<i>H. vulgare</i> IFAM MC-750	<i>H. aestuarii</i>	<i>H. chloromethanicum</i>	<i>H. coagulans</i>	<i>H. denitrificans</i>	<i>H. facile</i> subsp. <i>facile</i>	<i>H. facile</i> subsp. <i>tolerans</i>	<i>H. facile</i> subsp. <i>ureaphilum</i>	<i>H. hollandicum</i>	<i>H. methylovorum</i>	<i>H. zavarzinii</i>
Mother cells oval, pear-, or drop-shaped	+	+	—	+	+	+	+	+	+	+	+	+
Mother cells bean-shaped	—	—	+	+	—	—	—	—	—	—	—	—
Mother cell 0.5–1.2 µm wide	+	+	+	+	+	—	+	+	+	+	—	+
Mother cell 0.3–0.65 µm wide	—	—	—	—	—	+	—	—	—	—	+	—
Mother cell average length, µm	1–3 ^c	2	1.64	1.3–1.8	1.2–2.0	1.0–3.0	2	1.93	1.9	1.66	0.5–1.2	1.78
Polar flagella	nd	—	—	nd	+	nd	—	—	—	—	—	—
Lateral flagella	nd	—	—	nd	—	nd	—	—	—	—	+	—
Subpolar flagella (1-3)	nd ^d	+	+	nd ^d	—	nd	+	+	+	+	—	+
Mother cells with polar holdfast	—	—	—	nd	—	nd	—	—	—	—	—	+
Rosette formation (R) or cell clumping (C)	C	C	C	—	C	C	—	—	—	C	—	R
Growth as turbidity (T) or pellicle (P)	T	T	P	T	P	T	T	T	T	v	T	v
Older cells yellow	—	—	—	—	+	—	—	—	—	—	+	—
Isolated from soil (S), water (W), or sewage (SW)	S	S	W	S	S	S	S	S	S	SW	S	S

^aSymbols: —, 90% or more of strains are negative; +, 90% or more of strains are positive; v, variable, depending on growth conditions; nd, not determined.

^bStrains: *H. vulgare* Stutzer and Hartleb 1898; *H. vulgare* IFAM MC-750; *H. aestuarii* IFAM NQ-521Gr; *H. chloromethanicum* CM2; *H. coagulans* 10-2; *H. denitrificans* TK 0415; *H. facile* subsp. *facile* IFAM H-526; *H. facile* subsp. *tolerans* IFAM I-551; *H. facile* subsp. *ureaphilum* IFAM CO-582; *H. hollandicum* IFAM KB-677; *H. methylovorum* KM-146; *H. zavarzinii* IFAM ZV-622.

^cFrom stained preparations.

^dMotility was observed.

TABLE BXII.α.164. Physiological properties of species of the genus *Hyphomicrobium*^a

Characteristics	<i>H. vulgare</i> ^b	<i>H. vulgare</i>	<i>H. aestuarii</i>	<i>H. chloromethanicum</i>	<i>H. coagulans</i>	<i>H. denitrificans</i>	<i>H. facile</i> subsp. <i>facile</i>	<i>H. facile</i> subsp. <i>tolerans</i>	<i>H. facile</i> subsp. <i>ureaphilum</i>	<i>H. hollandicum</i>	<i>H. methylovorum</i>	<i>H. zavarzinii</i>
<i>C</i> -source:												
Acetate	+	+	+	nd	—	+	(+)	—	—	—	—	(+)
<i>n</i> -Butyrate	nd	+	(+)	nd	nd	—	—	+	+	—	nd	+
Lactate	+	—	+	nd	—	—	+	—	—	+	—	—
Succinate	+	+	+	nd	nd	—	—	—	—	+	—	—
Chloromethane	nd	—	—	+	—	nd	—	—	—	—	nd	—
Ethanol	nd	—	+	+	+	(+)	(+)	—	(+)	—	—	+
Glycerol	+	+	+	nd	nd	—	+	+	+	+	—	+
Amygdalin	nd	+	—	nd	nd	nd	(+)	—	—	+	nd	(+)
Peptones	—	—	+	nd	+	—	—	+	—	—	nd	+
<i>N</i> -Acetylglucosamine	nd	+	(+)	nd	nd	nd	+	+	(+)	+	nd	—
Formamide	nd	—	+	nd	nd	nd	—	—	—	—	+	—
Aspartate	nd	—	—	nd	+	nd	—	—	—	+	+	+
Asparagine	+	(+)	(+)	nd	nd	nd	—	—	—	—	nd	—
Oligocarbophilic	+	+	+	+	nd	nd	+	+	+	+	—	+
Nitrate reduction	+ ^c	+	+	—	—	+	—	—	—	—	—	+
<i>Growth at:</i>												
5°C	nd	—	—	nd	nd	nd	+	—	—	—	—	—
15°C	+	+	+	nd	nd	nd	+	+	+	—	+	+
37°C	nd	+	+	nd	nd	—	+	+	+	+	—	+
45°C	nd	—	+	nd	nd	—	—	+	+	—	—	—
Maximum pH	nd	>7.0	>7.0	7.5	~6.0	>7.0	>7.0	>7.0	>7.0	>7.0	>7.0	>7.0
Inhibition by visible light	nd	—	+	nd	nd	nd	—	—	—	+	nd	+
Growth stimulated by B ₁₂	nd	—	—	—	nd	nd	+	—	—	—	+	—
Gelatin liquefaction	—	—	—	nd	+	—	—	—	—	—	—	—
Milk coagulation	nd	—	—	nd	+	nd	—	—	—	—	nd	—
H ₂ evolution	nd	—	—	nd	+	nd	—	—	—	—	—	—
α-Hemolysis of sheep blood	nd	+	—	nd	nd	nd	—	—	+	—	nd	+
Genome size: mol. wt. (× 10 ⁹) ^d	nd	2.13	2.62	nd	nd	nd	nd	nd	nd	2.43	nd	nd
Mol% G + C of DNA (<i>T_m</i>)	nd	61	64	60	nd	60–61	59	59	60	62	61	65

^aSymbols: —, 90% or more of strains are negative; +, 90% or more of strains are positive; nd, not determined; (+), growth stimulation weak but significant.

^bStrains: *H. vulgare* Stutzer and Hartleb 1898; *H. vulgare* IFAM MC-750; *H. aestuarii* IFAM NQ-521Gr; *H. chloromethanicum* CM2; *H. coagulans* 10-2; *H. denitrificans* TK 0415; *H. facile* subsp. *facile* IFAM H-526; *H. facile* subsp. *tolerans* IFAM I-551; *H. facile* subsp. *ureaphilum* IFAM CO-582; *H. hollandicum* IFAM KB-677; *H. methylovorum* KM-146; *H. zavarzinii* IFAM ZV-622.

^cAnaerobic growth with KNO₃, but nitrate reduction was considered to be negative.

^dData from Kölbel-Boelke et al. (1985).

tory. The type strain grew well with propionate, isobutyrate, valerate, and mannitol. It did not grow with fructose or sucrose.

Nitrogen sources utilized (in order of growth stimulation) are: NH₄⁺, NO₃[—], NO₂[—], and ureate. Do not use urea or fix N₂, although slow oligonitrophilic growth has been observed. Do not nitrify. Anaerobic growth occurs in the presence of NO₃[—], but NO₂[—] has not been detected. Slow growth on sheep blood agar.

Strain IFAM MC-750 is inhibited by 30 µg disks of kanamycin, neomycin, and novobiocin and by 10 µg of streptomycin. It tolerates 5.5% NaCl, but growth is retarded at this concentration. The pH optimum is between 6.5 and 7.5; the temperature range for growth is 15–37°C. IFAM MC-750 is catalase and cytochrome oxidase positive, sheep's blood is hemolyzed (α-hemolysis), and most cells form poly-β-hydroxybutyrate as a storage product. MC-750 is not pathogenic for mice and guinea pigs.

The genome size is 2.13 × 10⁹ Da (Kölbel-Boelke et al., 1985).

Habitat: soil. The neotype strain came from construction soil.

The mol% G + C of the DNA is: 61.4 (*T_m*) (Gebbers et al., 1986) or 61.1 (HPLC) (Urakami et al., 1995b).

GenBank accession number (16S rRNA): Y14302.

Additional Remarks: IFAM MC-750 (ATCC 27500) is recommended as the neotype strain; the original type strain no longer exists.

- 2. *Hyphomicrobium aestuarii*** Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1901)
ae.stu.a'ri.i. L. n. *aestuarium* estuary; M.L. gen. n. *aestuarii* of the estuary.

Mother cells bean-shaped, often with short hyphae; the terminal bud on a hypha is also bean shaped, but is turned at a 90° angle from the mother cell. Mother cells 0.6 ×

1.6 μm (range: $0.5\text{--}1.0 \times 0.6\text{--}5.0 \mu\text{m}$). Older hyphae branched. Swarmer cells with 1–3 subpolar flagella. Cells do not form rosettes, but clump easily and grow in liquids as surface pellicles; shaking precipitates the pellicle. Growth also occurs on the glass surface near the top of the vessel. Attachment of swarmer cells to the glass walls is inhibited by light. Colonies on solid media are brownish, strongly and irregularly folded; surface often has concentric rings. Colony cells very cohesive.

Chemoorganotrophic, aerobic, oligocarbophilic. Grow well with methanol, methylamine·HCl, formate, formamide, dilute human urine, acetate, pyruvate, malate, ethanol, and acetamide. Growth is stimulated, but slow, with *n*-butyrate, isovalerate, lactate, α -oxoglutarate, succinate, crotonate, β -hydroxybutyrate, oxalate, glucuronate, ethanol, *n*-propanol, isobutanol, formaldehyde, glycerol, chitin, and Bacto peptone. Also stimulatory (as C sources) are: D,L-aspartate, L-asparagine, and N-acetylglucosamine.

Nitrogen sources utilized for growth: NH_4^+ , NO_3^- , and urea; the organisms are oligonitrophilic. Slow growth on Bacto peptone with methylamine·HCl added and on sheep blood agar, but there is no hemolysis.

Inhibited by 30 μg each of kanamycin, neomycin, tetracycline, and erythromycin and by 10 μg of streptomycin (all administered on disks). Grow well in the presence of 3.5% NaCl and faintly with 5.5% NaCl. Temperature range: 5–45°C. Optimal pH: 6.5–7.5. Inhibited by visible light. With methanol and KNO_3 , there is anaerobic growth and gas formation; grow anaerobically with methylamine in the presence of thioglycolate. Cytochrome oxidase and catalase positive; gelatin is not liquefied. Not pathogenic for mice or guinea pigs.

Habitat: isolated from brackish water of the Elbe River estuary near Cuxhaven, Germany (Mevius, 1953) and from harbor water of Woods Hole, Massachusetts (Hirsch and Rheinheimer, 1968). Originally present in enrichments of nitrifiers.

The genome size is 2.62×10^9 Da (Kölbel-Boelke et al., 1985).

The mol% G + C of the DNA is: 64.1 (T_m) (Gebers et al., 1986) or 65.5–66.1 (HPLC) (Urakami et al., 1995b; Urakami and Komagata 1987b).

Type strain: ATCC 27483, IFAM NQ-521Gr.

Additional Remarks: Additional strains include IFAM MEV-533, IFAM MEV-533Gr (ATCC 27488), IFAM WH-563, IFAM EA-617, IFAM EN-616, IFAM NQ-521, and IFAM NQ-528. With the exception of strain IFAM WH-563, all of these strains are descendants of isolate "B", which was originally obtained by Mevius (1953).

3. **Hyphomicrobium chloromethanicum** McDonald, Doronina, Trotsenko, McAnulla and Murrell 2001, 121^{VP}
chlo.ro.me.tha'ni.cum. Gr. adj. *chloros* green, referring to the chlorine radical; M.L. adj. *methanicus* methane (utilizing); N.L. neut. n. *chloromethanicum* chloromethane (utilizing).

Monoprosthecae rods, budding on the prosthecal (hyphal) tips. Cells $0.5\text{--}0.6 \times 1.3\text{--}1.8 \mu\text{m}$, nonpigmented, may store poly- β -hydroxybutyrate. Daughter cells are motile. Growth is aerobic. Cells are restricted facultative methylo-trophs, capable of growth on one-carbon compounds, such as chloromethane, methanol, or methylamine as sole sources of carbon and energy. The chloromethane-degrad-

ing enzymes are inducible. Growth occurs also on ethanol. The type strain, CM2^T, also oxidizes, but does not grow on, bromomethane or iodomethane. Do not grow autotrophically, but growth occurs on mineral salts medium or mineral agar (pH 7.2) with 1% (v/v) CH_3Cl as gas; pyruvate-dehydrogenase activity is negative. C_1 compound assimilation is via the serine pathway (icl^+); NH_4^+ assimilation is via the glutamate cycle. Nitrate is not reduced; oxidase and catalase activity is positive. Vitamins are not required. Storage of viable cells on Whatman paper in sealed flasks or ampules kept at -40 to -80°C (Doronina and Trotsenko, 1992).

The temperature optimum for growth is $28\text{--}30^\circ\text{C}$. The pH optimum for growth is 6.5–7.5. The major fatty acid is $\text{C}_{18:1}$ and the major quinone is Q-9.

H. chloromethanicum shares high 16S rRNA gene sequence similarity with *H. facile* subsp. *facile* (97.1%), *H. denitrificans* (96.3%), and *H. methylivorum* (96.0%). The DNA–DNA similarity with *H. zavarzinii* ZV-622^T has been found to be 29.4%.

Habitat: strain CM2 was isolated from polluted soil at the Nizhekamsk petrochemical factory, Tatarstan, Russia. Strain CM29 came from soil of the Alushta dendropark, Crimea, Ukraine.

The mol% G + C of the DNA is: 60 (T_m).

Type strain: CM2, NCIMB 13687, VKMB-2176.

GenBank accession number (16S rRNA): AF198623.

Additional Remarks: CM29 is identical to CM2.

4. **Hyphomicrobium coagulans** (ex Takada 1975) Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1903.)
co.a'gu.lans. L. part. adj. *coagulans* curdling, coagulating.

Mother cells ovoid or pear-shaped, $0.6\text{--}1.2 \times 1.2\text{--}2.0 \mu\text{m}$. Hyphae polar or bipolar, branched in older cultures. Swarmer cells motile with 1–3 polar flagella. In liquid media, growth occurs as turbidity or pellicle. Growth on solid media may become yellow.

Chemoorganotrophic, aerobic. Good growth on methanol, methylamine, and ethanol. Other carbon sources utilized are: *n*-propanol, *n*-butanol, isobutanol, benzyl alcohol, furfuryl alcohol, trimethylene glycol, formate, and citrate. Carbon sources not utilized: acetate, lactate, formaldehyde, and glycerate. Growth is stimulated by glucose, sucrose, and lactose. Methane is utilized as a carbon source.

Acid is formed (without gas) from glucose, lactose, glycerol, methyl- α -glucoside, sucrose, maltose, salicin, arabinose, galactose, mannitol, inulin, xylose, and soluble starch. Gelatin is liquefied, peptone is utilized, and milk is slowly coagulated, but not peptonized. Casein is not hydrolyzed, indole is not produced. H_2S -positive; methyl red test and Voges–Proskauer tests are positive. Nitrate reduction negative.

Nitrogen sources utilized are: Casamino acids, polypeptone, aspartate, KNO_3 , and NH_4^+ .

Optimal pH: ~ 6.0 ; range: 5.7–8.0. Temperature optimum: 35°C .

Habitat: rice field soil of Hirakata, Osaka, Japan.

The mol% G + C of the DNA is: not reported.

Type strain: Takada 10-2.

5. **Hyphomicrobium denitrificans** Urakami, Sasaki, Suzuki and Komagata 1995b, 531^{VP}
de.ni.tri'fi.cans. M.L. part. adj. *denitrificans* denitrifying.

Mother cells are rod-shaped, oval, egg-shaped or bean-shaped, often with pointed ends, $0.3\text{--}0.6\text{ }\mu\text{m} \times 1.0\text{--}3.0\text{ }\mu\text{m}$, with mono- or bipolar hyphae of varying lengths that are $0.3\text{--}0.4\text{ }\mu\text{m}$ in diameter. Swarmer cells motile, often attaching to surfaces or to other cells and forming clumps. Granules of poly- β -hydroxybutyrate accumulate in the cells.

Colonies on methanol-containing agar are shiny, smooth, raised, entire, white, and 1–2 mm after 3–6 d at 30°C . Chemoorganotrophic, aerobic, not fermentative. Good growth on methanol, mono-, di-, and trimethylamine, pectin, and acetate. Methanol is utilized by the serine pathway via activated formaldehyde incorporation.

Some strains utilize formate and ethanol. The following compounds are not utilized: L-arabinose, D-xylose, D-glucose, D-mannose, galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol, soluble starch, propionic acid, isobutyric acid, *n*-valeric acid, lactic acid, succinic acid, oxalic acid, and methane. Vitamins and amino acids are not required for growth. No growth on nutrient broth or peptone broth.

Nitrogen sources utilized: ammonia, nitrate, and urea. Nitrate is reduced to nitrite; nitrate reduction is strong. Methyl red, Voges-Proskauer, gelatin, and starch hydrolysis are negative. Indole, H_2S and ammonia are not produced. Oxidase, urease, and catalase positive. Acid is not produced from sugars oxidatively or fermentatively.

Optimal pH range for good growth: pH 6.0–8.0. Good growth at 30°C . No growth in nutrient broth, peptone broth, or in media with 3% sodium chloride.

The cellular fatty acids include large amounts of straight-chain, unsaturated $\text{C}_{18:1}$ acid. The hydroxy fatty acids include large amounts of $\text{C}_{14:0\ 3\text{OH}}$ and $\text{C}_{16:0\ 3\text{OH}}$ hydroxy acids. The major ubiquinone is Q-9.

Habitat: soil.

The mol% G + C of the DNA is: 60.4 (HPLC) (Urakami et al., 1995b) or 60.5 (HPLC) (Urakami and Komagata, 1987b).

Type strain: Attwood and Harder strain X, DSM 1869, NCIB 11706, TK 0145.

GenBank accession number (16S rRNA): Y14308.

6. **Hyphomicrobium facile** Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1902.)

fa'ci.le. L. adj. *facile* ready, quick.

The three subspecies of *Hyphomicrobium facile* are described below.

The mol% G + C of the DNA is: 59.5–61.2

Type strain: see subspecies list below.

GenBank accession number (16S rRNA): see subspecies list below.

a. **Hyphomicrobium facile** *subsp. facile* Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1902.)

Mother cells pear- or drop-shaped, $0.95 \times 2.0\text{ }\mu\text{m}$ (range: $0.6\text{--}1.5 \times 0.9\text{--}6.0\text{ }\mu\text{m}$); on some media, hyphae are richly branched and often formed bipolarly. Swarmer cells have 1–3 subpolar flagella, which are easily shed. Grow in liquid media as turbidity; colonies on solid media are light brownish to beige, with a smooth and shiny surface and entire edges. Vigorous stirring of liquid cultures results in intercalary bud formation in hy-

phae. Chemoorganotrophic, aerobic. Grow well with methanol, methylamine·HCl, *N*-acetylglucosamine, and gelatin (strain IFAM B-522). Growth is significantly stimulated by formate, acetate, lactate, α -oxoglutarate, succinate, β -hydroxybutyrate, oxalate, glucuronate, ethanol, glycerol, amygdalin, chitin, and poly- β -hydroxybutyrate. Oligocarbophilic. Growth is also stimulated significantly by DL-leucine, DL-lysine, and DL-phenylalanine. There is no growth in dilute human urine.

Nitrogen sources utilized: NH_4^+ , NO_3^- , urea, and (slow) Bacto peptone. Slow growth oligonitrophilically. There is faint growth, but no hemolysis, on sheep blood agar. Inhibited by 30 μg each of kanamycin, neomycin, tetracycline, and erythromycin and by 10 μg streptomycin. Slow growth in the presence of 3.5% NaCl. Temperature range: $5\text{--}37^\circ\text{C}$. Optimal pH: 6.5–7.0. Light does not inhibit growth. Grow anaerobically with methylamine·HCl and thioglycolate; denitrification-negative; catalase and cytochrome oxidase positive. Gelatin is not liquefied, and poly- β -hydroxybutyrate is formed as a storage product. Lysine decarboxylase negative.

Not pathogenic for mice or guinea pigs.

The genome size is 2.35×10^9 Da (strain IFAM B-522).

Habitat: soil.

The mol% G + C of the DNA is: 59.5–61.2 (T_m , HPLC) (Gebers et al., 1986; Urakami et al., 1985, 1995b).

Type strain: ATCC 27485, IFAM H-526.

GenBank accession number (16S rRNA): Y14309.

Additional Remarks: Additional strains include IFAM D-524, E-525, G-527, K-529, L-530, and B-522 (ATCC 27484).

b. **Hyphomicrobium facile** *subsp. tolerans* Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1902.)

to'le.rans. L. part. adj. *tolerans* tolerating; pertaining to the tolerance of high CO concentrations.

Morphology and cell sizes as in *H. facile* *subsp. facile*. Colonies on solid media are light brown to beige, smooth and shiny, with entire edges. Grow in liquids as turbidity.

Chemoorganotrophic, aerobic, oligocarbophilic. Good growth on methanol, methylamine·HCl, formate, *n*-butyrate, isovalerate, glycerol, and *N*-acetylglucosamine. Slow growth, but significant stimulation, by pyruvate, crotonate, β -hydroxybutyrate, isobutanol, formaldehyde, Bacto peptone, and ureate. Nitrogen source utilized: NH_4^+ . Slow growth, but not hemolysis, on sheep blood agar. Inhibited by 30 μg each of kanamycin, neomycin, tetracycline, and erythromycin and by 10 μg of streptomycin. Growth in the presence of 2.5% NaCl has been observed; growth is faint with 5.5% NaCl. Temperature range: $15\text{--}45^\circ\text{C}$. Optimal pH: 6.5–7.5. Not inhibited by visible light. Do not denitrify, but growth occurs with methylamine·HCl anaerobically with thioglycolate. Catalase and cytochrome oxidase positive. Tolerate up to 90% (v/v) of CO in the atmosphere. Gelatin is not liquefied; poly- β -hydroxybutyrate is formed. Not pathogenic for mice or guinea pigs.

Habitat: soil.

The mol% G + C of the DNA is: 59.4–60.5 (T_m , HPLC); (Urakami et al., 1985; Gebers et al., 1986; Urakami and Komagata, 1987b).

Type strain: ATCC 27489, IFAM I-551.

GenBank accession number (16S rRNA): Y14311.

Additional Remarks: Additional strains include IFAM O-545, P-546, Q-547, R-549, M-552, CO-553, CO-557, and CO-558 (ATCC 27491).

- c. **Hyphomicrobium facile** *subsp. ureaphilum* Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1902.)
u.re.a.phil'um. Gr. n. *urum* urine; Gr. adj. *philus* loving; M.L. adj. *ureaphilum* loving urea.

Cell sizes and morphology are very similar to IFAM I-551 (Fig. BXII.α.189). Mother cell width 1.1 µm (range: 1.0–1.25 µm). Grow as turbidity in liquid media. Colonies on solid media brownish to beige, smooth, shiny, with entire edges. Physiological properties as for *H. facile* *subsp. tolerans* IFAM I-551, except for the following: growth is stimulated significantly by α-oxoglutarate, but not by crotonate; growth is stimulated by ethanol, but not by Bacto peptone, urea, and ureate as carbon sources. Urea is utilized as a nitrogen source in the presence of methanol. Temperature range: 25–37°C; there is weak growth at 15°C and 45°C. Grow weakly on sheep blood agar with α-hemolysis. Poly-β-hydroxybutyrate may be formed.

Habitat: soil.

The mol% G + C of the DNA is: 60.5 (T_m , HPLC) (Urakami et al., 1995b; Gebers et al., 1986).

Type strain: ATCC 27492, IFAM CO-582.

GenBank accession number (16S rRNA): Y14310.

Additional Remarks: Additional strains include IFAM CO-573, CO-574, CO-587, CO-611, CO-613, CO-614, CO-610, and CO-645.

7. **Hyphomicrobium hollandicum** Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1902.)
hol.lan'di.cum. M.L. neut. adj. *hollandicum* pertaining to the isolation from the Netherlands.

Mother cells oval or pear-shaped, 0.70×1.66 µm (range: $0.6\text{--}0.8 \times 1.5\text{--}3.5$ µm). Older hyphae branched. Swarmer cells subpolarly flagellated. Growth in liquid media as pellicle or turbidity; cells may tend to clump. Colonies on solid media are brownish in transparent light and beige in reflected light, with entire edges and shiny surface. Chemoorganotrophic, aerobic, oligocarbophilic. Grow well with methanol, methylamine·HCl, formate, glycerol, cellobiose, raffinose, dextrin, amygdalin, esculin, *N*-acetylglucosamine, and ureate. Growth is stimulated with isovalerate, pyruvate, lactate, succinate, oxalate, isobutanol, formaldehyde, L-sorbose, D-maltose, chitin, DL-aspartate, D-glucosamine, and dilute urine.

Nitrogen sources utilized: NH_4^+ , NO_2^- , NO_3^- . The bacteria grow slowly without an added nitrogen source, i.e., oligonitrophilically. Strain IFAM KB-677 is inhibited by 30 µg each of kanamycin, neomycin, and tetracycline. There is no growth in the presence of 2.5% NaCl. Temperature range: 25–37°C. Optimal pH: >7.5. Strain KB-677 is slightly inhibited by visible light. Catalase and cytochrome oxidase positive, gelatin liquefaction and hemolysis negative. Poly-β-hydroxybutyric acid is formed as a storage product. Not pathogenic for mice or guinea pigs.

The genome size is 2.43×10^9 Da (Kölbel-Boelke et al., 1985).

Habitat: strain IFAM KB-677 was obtained from T.Y. Kingma-Boltjes, who isolated it from sewage.

The mol% G + C of the DNA is: 62.4 (T_m) (Gebers et al., 1986) or 62.9 (HPLC) (Urakami et al., 1995b).

Type strain: ATCC 27498, IFAM KB-677.

GenBank accession number (16S rRNA): Y14303.

Additional Remarks: Strain IFAM MC-651 (ATCC 27 497) is related and was isolated from soil by M. Macpherson-Kraviec.

8. **Hyphomicrobium methylovorum** Izumi, Takizawa, Tani and Yamada 1983, 439^{VP} (Effective publication: Izumi, Takizawa, Tani and Yamada 1982, 373.)

me.thy.lo.vo'rum. M.L. n. *methyl* the methyl radical; L. v. *voror* devour; M.L. n. *methylovorum* methyl devourer.

Mother cells oval, $0.3\text{--}0.65 \times 0.5\text{--}2.0$ µm, with monopolar or bipolar hyphae. Swarmer cells motile with a single lateral flagellum. On solid media, colonies white to faintly yellow, circular, convex, smooth, and glistening.

Chemoorganotrophic, aerobic, aminopeptidase positive. Carbon sources utilized for growth: methanol, methylamine, dimethylamine, and trimethylamine. Poor growth on formate and formamide. Compounds not utilized are: methane, formaldehyde, ethanol, *n*-propanol, glycerol, D-glucose, D-fructose, D-lactose, D-arabinose, sucrose, trehalose, melibiose, cellobiose, mannitol, inositol, dextrin, starch, acetate, pyruvate, lactate, succinate, oxalate, glycolate, glyoxylate, citrate, fumarate, malate, tartrate, glycine, and serine.

Nitrogen sources utilized: NH_4^+ and L-glutamine; poor growth on peptone, Casamino acids, L-cysteine, and L-aspartate. Nitrogen compounds not utilized: nitrate, urea, yeast extract, meat extract, glycine, and L-serine. Nitrate reduction, Voges-Proskauer, indole, H_2S , starch hydrolysis, citrate, and urease are all negative. Catalase positive. Temperature range for growth: 14–33°C. Optimal temperature: around 28°C. Optimal pH: around 7.0. Vitamins are not required.

Habitat: soil.

The mol% G + C of the DNA is: 60.6 (T_m) (Urakami et al., 1985) or 58.5 (HPLC) (Urakami et al., 1995b).

Type strain: DSM 5458, IFO 1480, KM-146.

GenBank accession number (16S rRNA): Y14307.

9. **Hyphomicrobium zavarzinii** Hirsch 1989b, 495^{VP} (Effective publication: Hirsch 1989, 1903.)

za.var.zin'i.i. M.L. gen. n. *zavarzinii* of Zavarzin, named for G.A. Zavarzin, the Russian microbiologist who isolated these bacteria.

Mother cells drop- or pear-shaped, somewhat slender, with hyphae that rarely branch. Mother cells 0.63×1.8 µm (range: $0.5\text{--}0.9 \times 0.7\text{--}2.5$ µm). Swarmer cells with 1–3 subpolar flagella. In liquid media under most growth conditions, rosettes are formed, since mother cells produce a polar holdfast. Growth in liquids initially as turbidity and later as a pellicle, with precipitation on the bottom. Colonies on solid media are colorless to light brownish or beige, smooth and shiny, with entire edges.

Chemoorganotrophic, aerobic, oligocarbophilic. Good growth with the following carbon sources: methanol, methylamine·HCl, formate, *n*-butyrate, isovalerate, crotonate, β-hydroxybutyrate, ethanol, *n*-propanol, isobutanol, and glyc-

erol. Growth is stimulated significantly by acetate, *n*-valerate, α -oxoglutarate, galacturonate, formaldehyde, D-glucose, D-mannose, D-melibiose, amygdalin, esculin, chitin, Bacto peptone, DL-lysine, DL-aspartate, and dilute human urine. Nitrogen sources utilized are: NH_4^+ , NO_2^- , NO_3^- , and (poorly) Bacto peptone. There is slow growth in the absence of added nitrogen sources (oligonitrophily). Poor growth on sheep blood agar with α -hemolysis. The following antibiotics inhibit growth at 30 μg (per disc): kanamycin, neomycin, and tetracycline. Streptomycin at 10 μg is also inhibitory. There is growth in the presence of 3.5% NaCl. Temperature range: 15–37°C. Optimal pH: 6.5–7.5. Visible light inhibits growth slightly.

Grow anaerobically with nitrate and gas formation (with methanol as the carbon source). With methylamine·HCl

and thioglycolate, there is little growth. Catalase and cytochrome oxidase are positive; gelatin liquefaction is negative. Poly- β -hydroxybutyrate is a storage product.

Not pathogenic for mice or guinea pigs.

Genome size: 2.73×10^9 Da (strain ZV-580; Kölbel-Boelke et al., 1985).

Habitat: peaty and moist soil near Moscow, Russia.

The mol% G + C of the DNA is: 61.8–64.8 (Bd, T_m , HPLC) (Mandel et al., 1972; Gebers et al., 1986; Urakami and Komagata, 1987b; Urakami et al., 1995b).

Type strain: ATCC 27496, IFAM ZV-622.

GenBank accession number (16S rRNA): Y14305.

Additional Remarks: Additional strains include IFAM ZV-580, ZV-620, MY-619, MC-625, MC-629, MC-630, and MC-627.

Genus II. *Ancalomicrobium* Staley 1968, 1940^{AL}

CHERYL JENKINS, PATRICIA M. STANLEY AND JAMES T. STALEY

An.ca'lo.mi.cro'bi.um. Gr. masc. n. *ancalos* arm; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. neut. n. *Ancalomicrobium* arm (-producing) microorganism.

Unicellular bacterium with conical cells ~1.0 μm in diameter. **Two to eight or more prosthecae** produced per cell. Prosthecae are cylindrical without crossbands and taper gradually from cell to a distal diameter of ~0.2 μm and a length of 2–5 μm when fully differentiated. Prosthecae may be bifurcated. **Budding bacterium**. Buds are formed directly from mother cell, never from prosthecae. Cells occur singly or in pairs prior to division; rarely form aggregates.

Gram negative. Flagella and holdfasts not produced. **Gas vacuoles** are formed by all strains investigated.

Facultatively anaerobic. Chemoheterotrophic. Use sugars anaerobically and aerobically. Ferments sugars by **mixed acid fermentation**. Some organic acids are used aerobically, but not fermented. Ammonium can be used as sole nitrogen source. Vitamins are required. Oxidase and catalase positive. Temperature range for type strain: 9–39°C. Optimal pH: 7.0, pH range 6.3–7.5. Found in freshwaters and pulp mill oxidation lagoons.

Type genus: *Ancalomicrobium adetum* Staley 1968, 1940.

FURTHER DESCRIPTIVE INFORMATION

A. adetum lives in freshwater habitats, including lakes and eutrophic habitats, such as ponds, rivers and pulp mill oxidation lagoons (Staley, 1971; Stanley et al., 1979; Staley et al., 1980).

The most distinctive feature of the genus is its cellular morphology. The size, shape, and location of the prominent prosthecae, combined with a lack of cell motility, are unique features of this genus. Under normal growth conditions, in which a low concentration of nutrients is supplied, the prosthecae attain a length of 2–5 μm (Fig. BXII.α.192). Shorter prosthecae, as formed by members of the genus *Prosthecomicrobium*, are not produced by *Ancalomicrobium*; however, aberrant cell morphology can be observed under *in vitro* cultivation conditions in the presence of high concentrations of nutrients.

Buds begin as small protuberances on a non-appendaged area

of the “mother cell”. The buds enlarge and differentiate, and new prosthecae are formed so that the daughter cell is a pseudo-mirror image of the mother cell at the time of cell separation. The mother cell retains its original prostheca, and each new bud is produced at the same location on the mother cell. The daughter buds are produced essentially *de novo* during the budding process (Staley, 1973b; Staley et al., 1981) as shown in Fig. BXII.α.193.

Gas vacuoles are produced by all strains studied. Gas vesicles may be produced in some cells under aerobic conditions with sugars as a carbon source; however, they are only observed during the stationary growth phase. In contrast, all strains studied produce gas vacuoles prodigiously during anaerobic growth at low temperatures (<18°C) (A. Van Neerven and J.T. Staley, unpublished observations). Gas vacuoles are also produced under microaerophilic conditions on organic acids, including acetic, pyruvic and succinic acids (R.L. Irgens and J.T. Staley, unpublished observations).

A variety of sugars, sugar alcohols, and organic acids can be used as carbon sources for aerobic growth. These include D-arabinose, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-galactose, D-xylose, L-fucose, L-rhamnose, D-melibiose, maltose, lactose, cellobiose, trehalose, N-acetylglucosamine, acetate, pyruvate malate, glycerol, inositol, and mannitol. Agar is digested slowly.

Some carbon sources, including some sugars and sugar alcohols, are used anaerobically by fermentation. Sugars are fermented by a mixed acid fermentation (Van Neerven and Staley, 1988). Glucose is fermented to acetic acid, ethanol, lactic acid, formic acid, succinic acid, hydrogen gas, and carbon dioxide. These are the same products that *Escherichia coli* produces under the same conditions for growth.

Ammonium salts are used as a sole source of nitrogen. Pantothenic acid is required for growth, and biotin, thiamine, folic

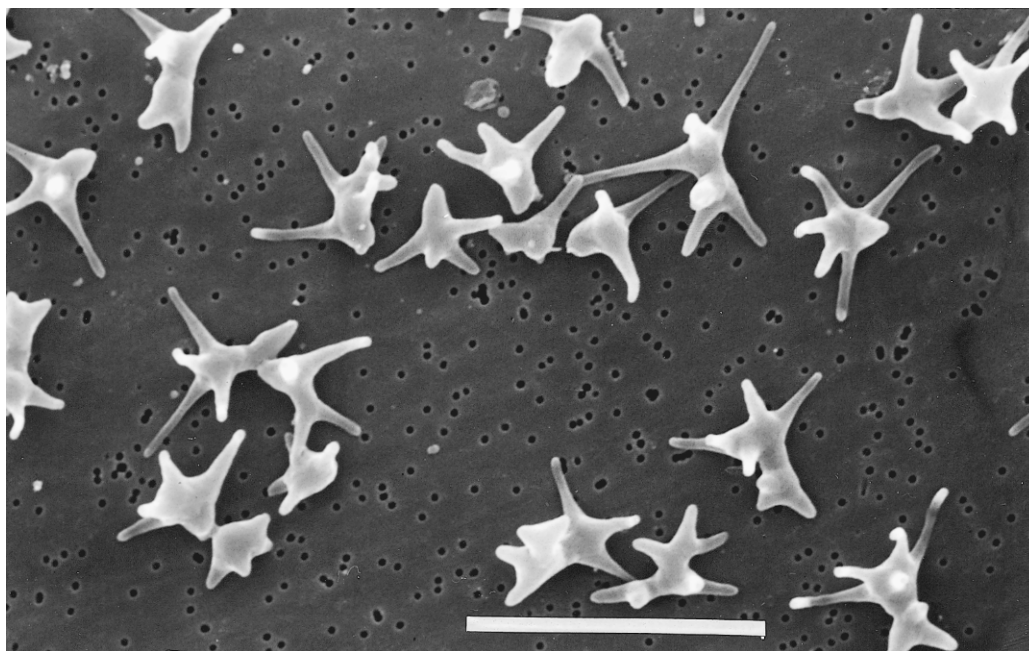


FIGURE BXII.α.192. *Ancalomicrobium adetum*. Scanning electron micrograph. Bar = 5.0 μm. (Courtesy of A.R.W. van Neerven.)

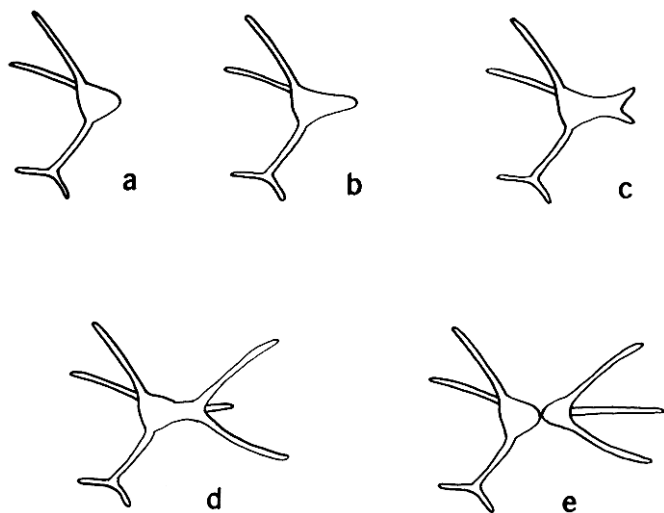


FIGURE BXII.α.193. A diagram of *Ancalomicrobium adetum*, illustrating the life cycle of this budding bacterium. The mother cell has three appendages, one of which is bifurcated (a). Bud formation occurs at the apex of the conical cell (b). The bud begins to differentiate (c and d) and ultimately separates from the mother cell (e). Following division, the mother cell, whose appearance after reproduction remains essentially identical to its initial appearance in a, will produce another daughter cell from the same location. This process will be repeated again and again, as long as conditions are favorable for growth. (Reproduced with permission from J.T. Staley, *Journal of Bacteriology* 95: 1921–1942, 1968, ©American Society for Microbiology.)

acid, and nicotinamide are stimulatory for growth of the type strain.

The type strain grows on a minimal, defined medium containing ammonium salts, phosphate, a modified Hutner's salts

solution, and vitamins (see *Bergey's Manual of Systematic Bacteriology*, first edition). Better growth is obtained in complex media, such as modified medium B (MMB; Staley, 1968), which contains yeast extract, in addition to the constituents provided in the defined medium. The highest yields have been obtained in a richer complex medium (R.L. Irgens, personal communication; Staley, 1989).

The minimal temperature for growth of the type strain is between 6 and 9°C, whereas the maximum lies between 39 and 43°C. The optimal pH for growth is between 6.9 and 7.3; however, growth has been observed at all pH values tested, ranging from 6.3 to 7.5.

ENRICHMENT AND ISOLATION PROCEDURES

Selective isolation procedures have not yet been developed. However, the addition of peptone to a final concentration of 0.01% may be used to enrich for these bacteria.

Enrichment cultures of 100 ml are normally used. Graduated 150-ml beakers containing 10 mg of peptone are covered with aluminum foil and autoclaved. Natural water samples are collected aseptically, and 100 ml aliquots are added to the beaker. Enrichment cultures are incubated at room temperature without shaking. Cultures should be examined periodically for the appearance of multiply appendaged prosthecae bacteria. Wet mounts should be prepared for phase microscopic observation, from the time the cultures become turbid (usually 4–7 days), until 2–3 weeks have elapsed. Whereas *Caulobacter* species are frequently observed attached to debris in the surface film of the enrichment, these planktonic multiply-appendaged forms are rarely encountered there. They are usually found moving with the currents in the wet mount and are most often seen in areas where free-floating forms have accumulated near debris or air bubbles. When multiply-appendaged forms comprise a significant portion of the enrichment culture (~1%), attempts should be made to isolate them by streaking onto MMB plates (see Staley,

1989). Alternatively, 10⁻³–10⁻⁵ dilutions of the enrichment culture can be spread-plated onto MMB agar. Plates should be incubated at room temperature for 1–2 weeks before examination. Individual colonies are selected for examination by wet mount. When positive colonies are found, they are restreaked for purification.

MAINTENANCE PROCEDURES

A. adetum can be maintained for at least 1 month on MMB slants that have been refrigerated. Long-term preservation is effected by lyophilization of cultures grown on complex media.

DIFFERENTIATION OF THE GENUS *ANCALOMICROBIUM* FROM OTHER GENERA

The striking morphology of members of the genus *Ancalomicrobium* allows them to be readily distinguished from nonappendaged bacteria and from most of the other prosthecae bacteria. However, some of the multiply-appendaged bacteria may appear similar. The genus *Stella* can be differentiated from *Ancalomicrobium* because all of its appendages lie in one plane. The most morphologically similar genus is *Prosthecomicrobium*. Some cells of *P. hirschii*, in particular, appear identical to *A. adetum*, although short-appendaged cells are also produced by *P. hirschii*. In addition, all *P. hirschii* strains are motile, while *A. adetum* is nonflagellated. Furthermore, *Ancalomicrobium* strains produce gas vacuoles, while *P. hirschii* does not. More significantly, these two groups are physiologically distinct. All species of *Prosthecomicrobium* are obligately aerobic, whereas *Ancalomicrobium* strains are fermentative, facultative anaerobes. Table BXII.α.165 summarizes the important differential characteristics that distinguish these two genera.

TAXONOMIC COMMENTS

DNA–DNA hybridization studies have shown that *Ancalomicrobium* strains and *Prosthecomicrobium* strains form separate taxonomic

groups (Moore and Staley, 1976). This finding is consistent with the current taxonomy of these two genera. According to 16S rRNA analyses, both *Ancalomicrobium* and *Prosthecomicrobium* species are members of the *Alphaproteobacteria*; however, based on sequence data from at least three strains, *Ancalomicrobium* has been shown to form a distinct genus (See Fig. BXII.α.194).

Although further taxonomic work on this genus is desirable, the difficulty encountered in obtaining isolates has hindered taxonomic investigations of the genus. However, since the initial isolation of the type strain, a number of strains have been isolated from pulp mill oxidation lagoons (A. Van Neerven and J.T. Staley, unpublished studies).

It is noteworthy that *A. adetum* is currently the only fermentative, heterotrophic, budding, and prosthecae bacterium in pure culture. Of special note in this regard is the discovery that *A. adetum* is a mixed acid fermenter whose fermentation products are identical to those of *E. coli* (Van Neerven and Staley, 1988). Furthermore, the phosphoenolpyruvate–sugar phosphotransferase system of this organism has been compared to that of certain enteric bacteria (Saier and Staley, 1977). The most interesting discovery from this investigation is that enzymatic cross-reactivity has been detected between the membrane-associated enzyme II complexes of *A. adetum* and the soluble enzyme I components of the enteric bacterium *Salmonella typhimurium*. It is noteworthy that *A. adetum* is a member of the *Alphaproteobacteria*, whereas the enteric bacteria are members of the *Gammaproteobacteria*.

FURTHER READING

Staley, J.T. 1989. Genus *Ancalomicrobium*. In Staley, Bryant, Pfennig and Holt (Editors), *Bergey's Manual of Systematic Bacteriology*, 1st Ed., Vol. 3, The Williams & Wilkins Co., Baltimore. pp. 1914–1916.
Staley, J.T. 1992. The genera: *Prosthecomicrobium*, *Ancalomicrobium* and *Prosthecoacter*. In Balows, Trüper, Dworkin, Harder and Schleifer (Editors), *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecology, Physiology, Isolation, Identification, Applications.*, 2nd Ed., Vol. III, Springer-Verlag, New York. pp. 2162–2166.

TABLE BXII.α.165. Differentiation of *Ancalomicrobium* from *Prosthecomicrobium*^a

Characteristic	<i>Ancalomicrobium</i>	<i>Prosthecomicrobium</i>
Short prosthecae (i.e., <2.0 μm when fully developed)	–	+
Long prosthecae (i.e., 2–5 μm when fully developed)	+	D ^b
Gas vesicles	+	D
Glucose fermentation	+	–

^aSymbols: –, no strains/species positive; +, all strains/species positive; D, varies depending on species.
^bSome species of *Prosthecomicrobium*, such as *P. hirschii* and *P. pneumaticum*, produce longer prosthecae as well as shorter ones.

List of species of the genus *Ancalomicrobium*

1. ***Ancalomicrobium adetum*** Staley 1968, 1940^{VP}
a'de.tum. M.L. adj. *adetum* arm or appendage.

The characteristics are as described for the genus. Occur in freshwater.
- The mol% G + C of the DNA is:* 70.4 (Bd)
Type strain: ATCC 23632, DSM 4722.

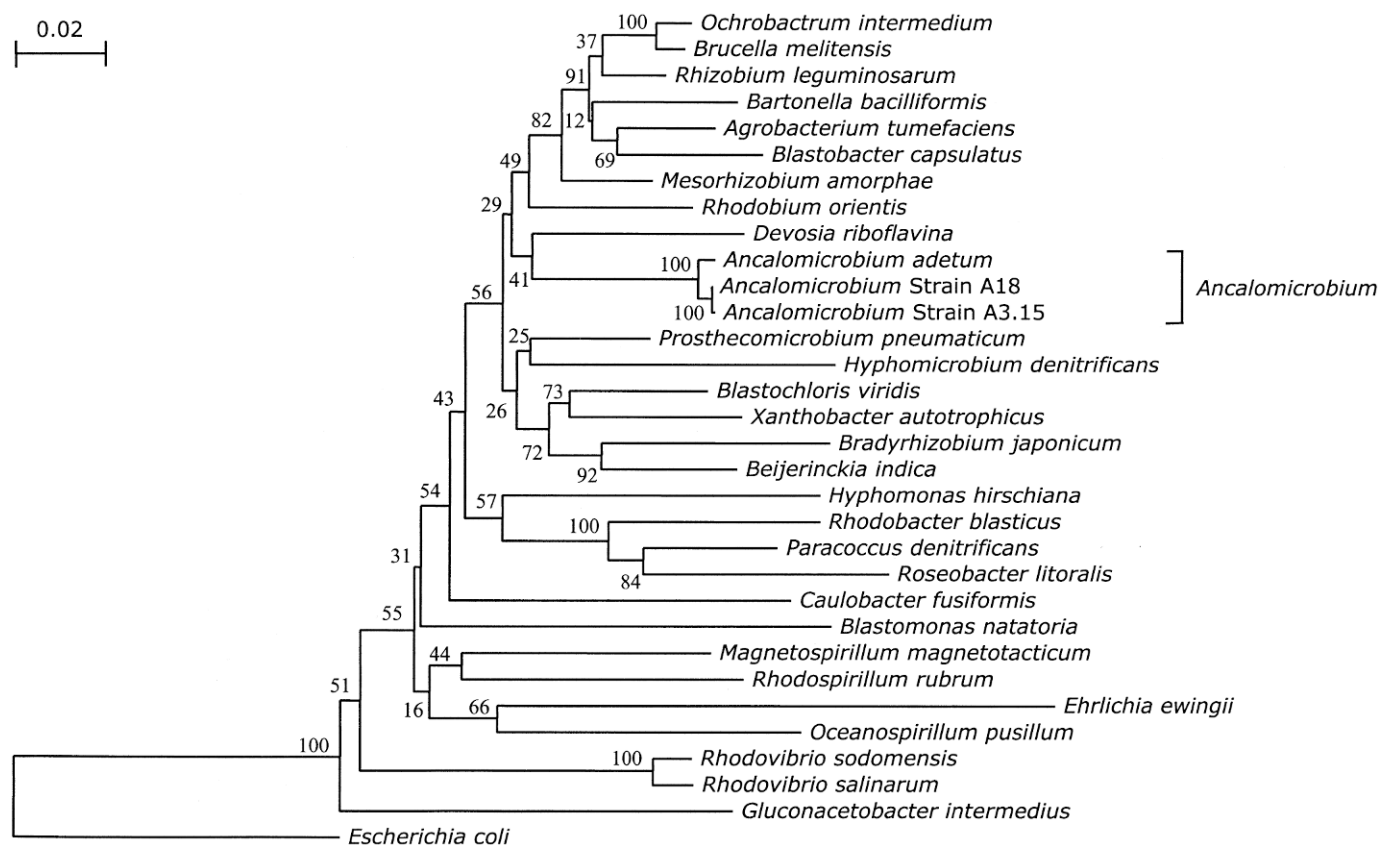


FIGURE BXII.α.194. Phylogenetic tree based on 16S rRNA sequences showing the position of *Ancalomicrobium* within the *Alphaproteobacteria*. The type strain of *Ancalomicrobium*, *A. adetum*, and two additional organisms isolated from pulp mill oxidation lagoons, strains A18 and A3.15, form a distinct genus. These strains form a separate lineage from other prosthecate bacteria, such as *Prosthecomicrobium* and *Hyphomicrobium*. The tree was constructed using Kimura distances and the neighbor-joining algorithm with 100 bootstrap replicates within the program Treecon. The distance scale indicates 0.02 substitutions per site.

Genus III. *Ancylobacter* Raj 1983, 397^{VP}

JAMES T. STALEY, CHERYL JENKINS AND ALLAN E. KONOPKA

An'cy.lo.bac'ter. Gr. adj. *ankylos* sharply curved; Gr. n. *bakterion* rod; M.L. masc. n. *Ancylobacter* a curved rod.

Curved rods, 0.3–1.0 × 1.0–3.0 μm. **Rings** (0.9–3.0 μm outer diameter) occasionally formed prior to cell separation. Coiled, helical, and filamentous forms are not produced. Cells are encapsulated. Resting stages not known. **Some strains produce gas vacuoles**. Gram negative. **Generally nonmotile**, but motility occurs in one gas vacuolate strain by means of a single polar flagellum. **Obligately aerobic**, possessing a strictly respiratory type of metabolism, with oxygen as the terminal electron acceptor. Optimal temperature, 22–37°C. Colonies are translucent to opaque and white to cream colored. Pellicles are produced in liquid media. **Oxidase positive**. Catalase positive. Chemoorganotrophic, using a variety of sugars or salts of organic acids as carbon sources. Chemolithotrophic growth has been reported on molecular hydrogen. Strains that have been tested can use methanol and formate (**facultatively methylotrophic**). Occur in soil and freshwater environments.

The mol% G + C of the DNA is: 66–69 (Bd).

Type species: *Ancylobacter aquaticus* (Ørskov 1928) Raj 1983, 297 (*Microcylus aquaticus* Ørskov 1928, 128.)

FURTHER DESCRIPTIVE INFORMATION

Cells of *Ancylobacter aquaticus* typically appear as curved rods (Figs. BXII.α.195 and BXII.α.196). Ring-like forms occur when the ends of a curved cell overlap prior to cell separation; this occurs infrequently under normal growth conditions. Pleomorphic forms have been reported under certain cultural conditions. For example, older cultures of the type strain contain swollen cells and other involution forms (Raj, 1970, 1977).

A number of gas-vacuolated strains of *Ancylobacter aquaticus* have been isolated (Nikitin, 1971; Van Ert and Staley, 1971; Konopka et al., 1977). These appear to be very similar to the type strain, so no new species have been proposed to accommodate them. We consider the type strain of *A. aquaticus* to be avacuolate, despite a claim to the contrary (Raj, 1977). In addition to a lack of convincing microscopic evidence for vacuoles in this strain in our laboratory, we have found that antiserum prepared against gas vacuoles is incapable of causing precipitation in lysates of the type strain, whereas precipitation does occur with the lysates of all gas-vacuolated strains.

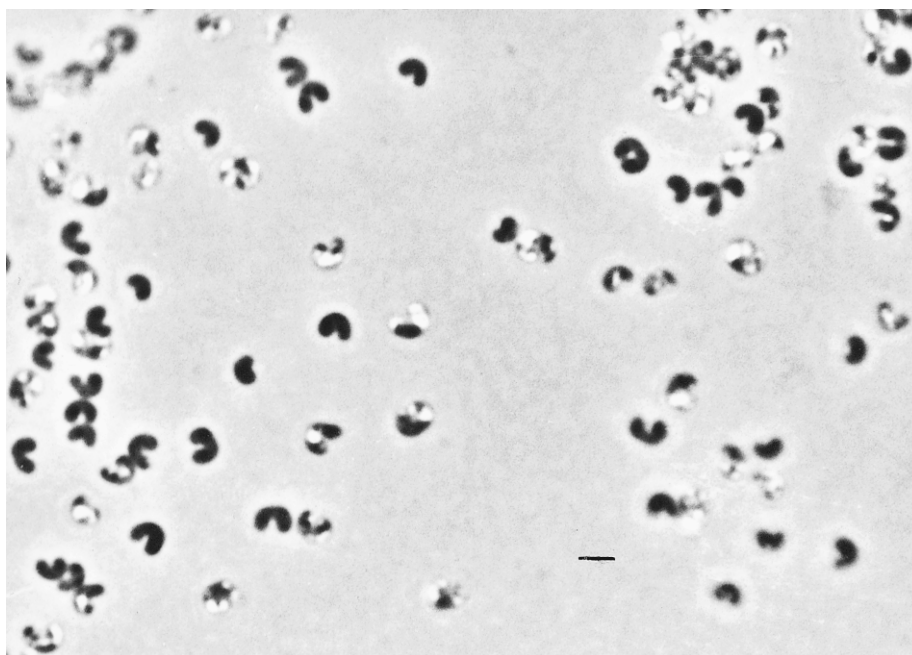


FIGURE BXII.α.195. A phase-contrast photomicrograph showing numerous cells of *Ancylobacter aquaticus*. The refractile intracellular areas of some cells are gas vacuoles. Bar = 2.0 μm.

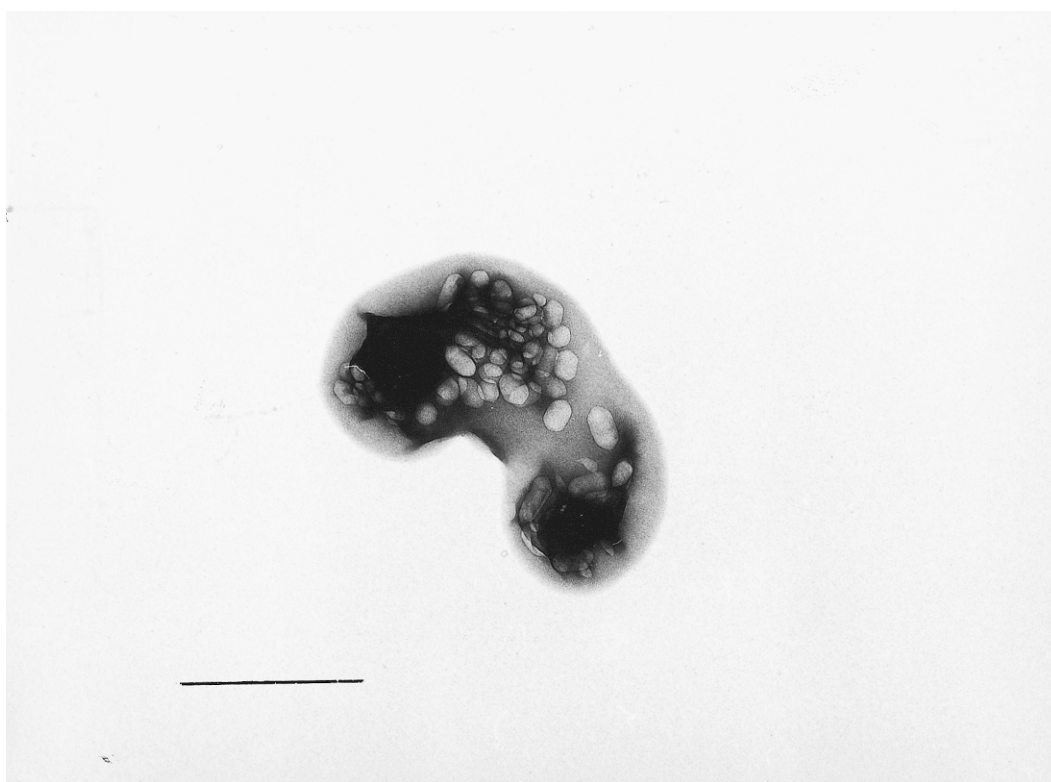


FIGURE BXII.α.196. An electron micrograph of a cell of a gas-vacuolated strain of *Ancylobacter aquaticus*. Individual gas vesicles can be seen. Bar = 1.0 μm. (Courtesy of Dr. J.C. Lara.)

Cells are encapsulated.

Motility has not been detected in *Ancylobacter*, except in the case of one of the gas-vacuolated strains of *A. aquaticus* (Konopka et al., 1976). In this strain (strain M6), the cells possess single polar flagellum. The occurrence of flagella is best seen in cells

cultivated at higher growth temperatures, i.e., above 20°C, a condition that precludes extensive gas vacuole formation. The strain forms numerous gas vacuoles when cultivated at 20°C, but flagella are rarely formed at this temperature.

The maximum temperature at which growth occurs is usually

37°C, although one strain can grow at 43°C. The minimum temperature for growth is 5°C.

Colonies of *Ancylobacter aquaticus* are nonpigmented to cream colored, circular, and convex, with an entire margin. In gas-vacuolated strains, the colonies appear in gradations from translucent (if few vacuoles are formed) to opaque, chalky white (if vacuoles are abundant).

Acid is produced from a variety of carbon sources when the medium of Hugh and Leifson (1953) is incubated aerobically (Raj, 1970; Van Ert and Staley, 1971; Larkin et al., 1977). All strains of *Ancylobacter aquaticus* form acid from arabinose, xylose, glucose, fructose, galactose, mannose, melibiose, sucrose, mannitol, sorbitol, and inulin. Gelatin is not liquefied. Starch is not hydrolyzed. Additionally, some of the strains, including the type strain, have been shown to produce acid oxidatively from glycerol and ribose, to reduce nitrate to nitrite and ammonia, to curdle and peptonize litmus milk by rendering it alkaline, and to possess catalase, β -galactosidase, lipase, ornithine decarboxylase, oxidase, and urease. However, they have been shown not to hydrolyze agar, chitin, casein, cellulose, or esculin, nor to produce indole, acetyl methyl carbinol, or H_2S (Raj, 1970; Larkin et al., 1977).

Strains of *Ancylobacter aquaticus* may be grown in a defined medium¹ containing an ammonium salt as a sole nitrogen source; individual carbon sources may be tested in this medium as sole carbon sources for growth. Lactate and pyruvate are used by all strains of *A. aquaticus* under these conditions. In addition, the type strain and some others utilize acetate, citrate, formate, gluconate, malonate, oxaloacetate, and succinate as sole carbon sources (Raj, 1977). Some strains of *A. aquaticus* are able to degrade chlorinated compounds, such as 1,2-dichloroethane (DCE), 2-chloroethanol, chloroacetate, and 2-chloropropionate, and use them as sole carbon and energy sources (van den Wijnngaard et al., 1992). All strains of *A. aquaticus* tested so far have been found to be facultative methylotrophs capable of utilizing C_1 compounds, such as methanol and formate (Van Ert and Staley, 1971; Namsaraev, 1973; Larkin et al., 1977). The one strain (Z-238) that has been analyzed for its enzyme content has been found to have ribulose biphosphate carboxylase activity when grown on methanol (Loginova et al., 1978). Due to induced dehydrogenases, methanol is oxidized sequentially to formaldehyde, formate, and finally carbon. Carbon dioxide is assimilated by ribulose biphosphate carboxylase and used in the Calvin cycle. Autotrophic growth of several strains has been achieved with hydrogen (Namsaraev and Nozhevnikova, 1978; Málek and Schlegel, 1981). A recent report indicates that some strains can grow as facultative chemolithoautotrophs using thiosulfate as an energy source (Stubner et al., 1998).

The pathways of glucose dissimilation have been determined by the use of position-radiolabeled glucose and gluconate (Kottel and Raj, 1973; Raj, 1977). In *Ancylobacter aquaticus* the Entner-Doudoroff pathway is the primary pathway and the pentose phosphate and Embden-Meyerhof-Parnas pathways are of lesser importance. In addition, a tricarboxylic acid cycle is present as a secondary pathway, as indicated by studies using specifically labeled acetate, glutamate, and pyruvate. The glyoxylic acid cycle does not appear to be operative in this species. Activities of key

enzymes from each of these pathways have been demonstrated by assays of cell extracts (Kottel and Raj, 1973).

Strains that have been tested grow well on nitrogen-free media (Nikitin, 1971; Málek and Schlegel, 1981). Attempts to demonstrate nitrogenase activity by the acetylene reduction method or by $^{15}N_2$ incorporation, however, have been unsuccessful (Málek and Schlegel, 1981).

The type strain of *Ancylobacter aquaticus* is sensitive to the following antibiotics (concentrations, μg /disk): ampicillin, 10; cephalothin, 30; chlortetracycline, 30; demeclocycline, 30; dihydrostreptomycin, 10; doxycycline, 30; erythromycin, 15; kanamycin, 30; neomycin, 30; nalidixic acid, 30; nitrofurantoin, 300; novobiocin, 30; oxytetracycline, 30; streptomycin, 5; tetracycline, 5 (Raj, 1970, 1977; Larkin et al., 1977).

Ancylobacter is not known to be pathogenic to humans.

Ancylobacter aquaticus occurs in freshwater habitats, including ponds, creeks, and lakes (Van Ert and Staley, 1971; Konopka et al., 1976), and in rice paddies and soil environments (Stubner et al., 1998). A number of strains have also been isolated from pulp mill oxidation lagoons (Konopka et al., 1976; Fulthorpe et al., 1993; Fulthorpe and Allen, 1995).

ENRICHMENT AND ISOLATION PROCEDURES

The original strain of *Ancylobacter aquaticus* was isolated on a medium consisting of agar (2%) and water (Ørskov, 1928). Gas-vacuolated strains can be isolated from enrichment cultures prepared with 100 ml of a freshwater source, such as creek water, added to a sterile aluminum foil-covered beaker containing 10 mg Bacto peptone (Difco). The enrichments are incubated at room temperature for 2 weeks, and dilutions are plated onto a hydrolysate medium² containing glucose (Van Ert and Staley 1971). These plates are incubated at 30°C for 1 week and examined for the development of chalky white colonies, which are indicative of gas vacuolate strains. Cells from such colonies should be observed by phase-contrast and electron microscopy to confirm that curved rods with refractile areas typical of gas vacuoles are in fact present.

MAINTENANCE PROCEDURES

Strains of *Ancylobacter aquaticus* are normally cultured on glucose-Casamino acids medium or TGEY medium.³ After incubation at 20–30°C to allow abundant growth, the cultures may be maintained in a refrigerator (5°C) for at least 3 weeks. They may also be preserved indefinitely by lyophilization.

DIFFERENTIATION OF THE GENUS *ANCYLOBACTER* FROM OTHER GENERA

Table BXII.α.166 provides the primary characteristics that can be used to differentiate this genus from morphologically similar, aerobic, nonmotile, nonphotosynthetic bacteria of the genera *Flectobacillus*, *Spirosoma*, and *Runella*. Two other genera of gas-vacuolated curved rods that may be confused with *Ancylobacter* are "*Brachyarcus*" and *Meniscus*. "*Brachyarcus*", which has never been isolated, differs from *Ancylobacter* in having cells arranged in groups (coenobia) consisting of two, four, or more rings (Skujala, 1964). *Meniscus* is differentiated from *Ancylobacter* by be-

1. Defined medium has the following composition (per liter of distilled water): $(NH_4)_2SO_4$, 0.25 g; glucose, 0.25 g (or molar equivalent of other carbon source); Na_2HPO_4 , 0.071 g; modified Hutner's salt solution, 20 ml; and vitamin solution, 10 ml. The salt solution is as described by Van Ert and Staley (1971), except that the amount of sodium molybdate is 12.67 mg. The vitamin solution is as described by Staley (1968).

2. Glucose-Casamino acids medium (per liter of distilled water): glucose, 1.0 g; Bacto Casamino acids (Difco), 1.0 g; modified Hutner's salt solution (see defined medium), 20 ml; vitamin solution (see defined medium), 10 ml; agar, 15.0 g.

3. TGEY medium is Bacto tryptone glucose extract agar (Difco) supplemented with 0.1% yeast extract (Raj, 1970).

TABLE BXII.α.166. Differentiation of *Ancylobacter* from morphologically similar heterotrophic genera

Property	<i>Ancylobacter</i>	<i>Spirosoma</i>	<i>Flectobacillus</i>	<i>Runella</i>
Shape	vibrios, rings	sinuous helices	large vibrios	curved rods
Colony pigmentation	none	yellow	pink	pink
Mol% G + C	66–69	51–53	39–41	49–50
Phylum:				
<i>Proteobacteria</i>	+			
<i>Bacteroidetes</i>		+	+	+

ing an aerotolerant anaerobe, rather than an aerobe, and by having a mol% G + C of 45 (Irgens, 1977). Furthermore, it is noteworthy that certain phototrophic bacteria, such as those of the purple nonsulfur genus *Rhodocyclus*, are morphological counterparts of *A. aquaticus*; however, *Rhodocyclus* species belong to a separate class of the *Proteobacteria*.

TAXONOMIC COMMENTS

Significant changes have occurred in the taxonomy of the genus *Ancylobacter*. In the previous edition of *Bergey’s Manual of Systematic Bacteriology*, the former genus name *Microcylus*, which is on the Approved List of names, was still in use. Based on morphological features, *Ancylobacter* was placed in Section 3 of the *Manual*, en-

titled “Nonmotile (or Rarely Motile), Gram-Negative Curved Bacteria”, along with, but not included in, a family of bacteria called the *Spirosomaceae* (Larkin and Borrall, 1978). Genera placed in the family *Spirosomaceae* were *Spirosoma*, *Runella*, and *Flectobacillus*. Subsequent phylogenetic analyses based on 16S rRNA sequences have shown that all genera of the *Spirosomaceae* are members of the *Flavobacterium–Cytophaga–Bacteroides* group, whereas *Ancylobacter* are members of the *Alphaproteobacteria* (Woese et al., 1990). The genus *Ancylobacter* forms part of the *Blastochloris viridis* subgroup of the *Alphaproteobacteria*, with *Starkeya novella* (formerly *Thiobacillus novellus*) as its closest relative (see Fig. BXII.α.197). Bacteria related to *Ancylobacter*, including members of the genera *Starkeya*, *Xanthobacter*, and *Blastochloris* (formerly *Rhodopseudo-*

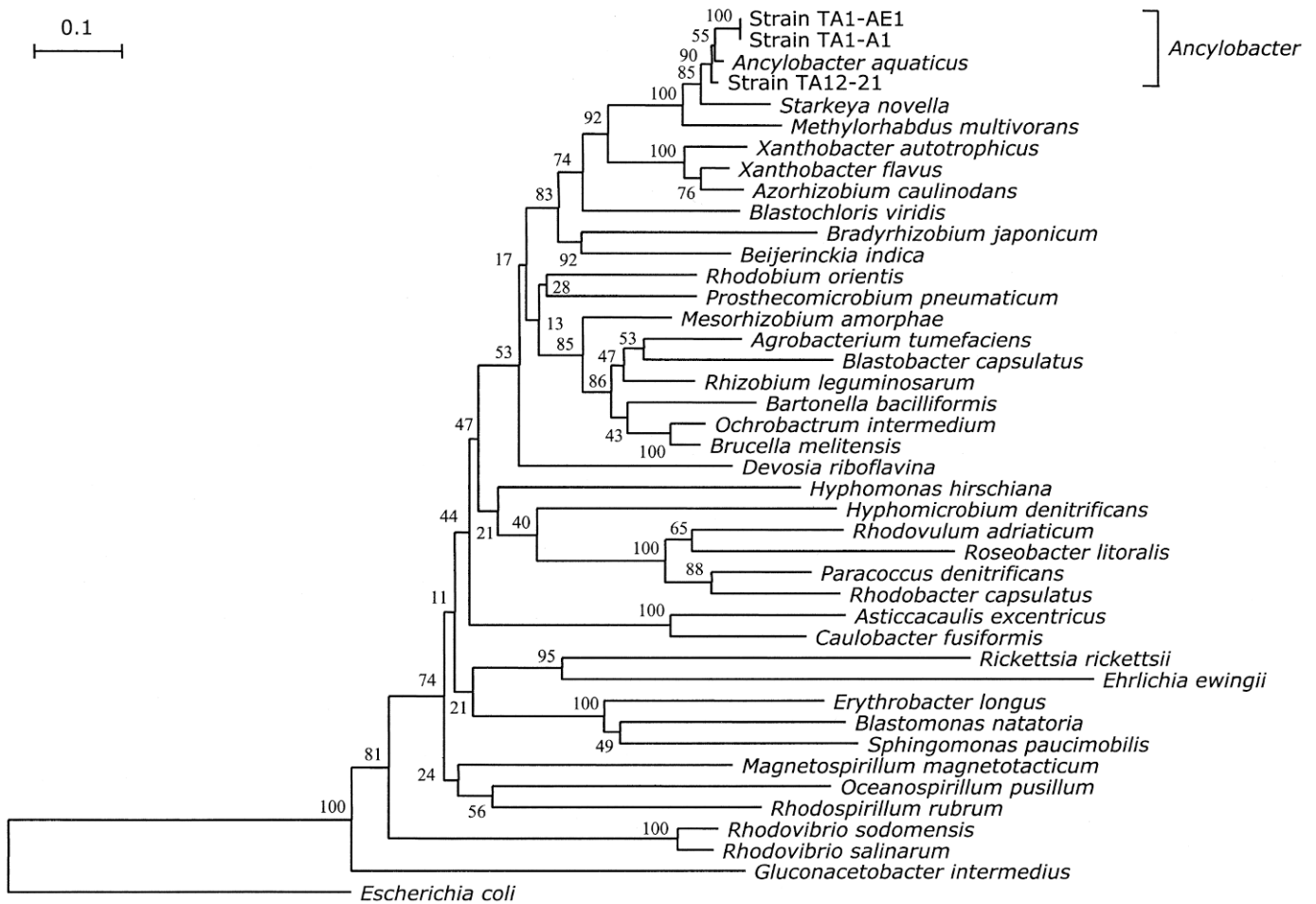


FIGURE BXII.α.197. Phylogenetic tree, based on 16S rRNA sequences, showing the position of *Ancylobacter* within the *Alphaproteobacteria*. Displayed in the tree are the type strain of *Ancylobacter aquaticus* and three additional strains, TA1-AE1, TA1-A1, and TA12-21, which were isolated from rice paddy soil. *Ancylobacter* forms part of the *Blastochloris viridis* subgroup of the *Alphaproteobacteria*, with the genera *Starkeya*, *Methylobacterium*, and *Xanthobacter* as the closest relatives. The phylogenetic tree was constructed using Kimura distances, the neighbor-joining algorithm, and 100 bootstrap replicates within the program Treecon. The distance scale indicates 0.1 substitutions per site.

monas), are also physiologically similar to *Ancylobacter* in that they can grow autotrophically. In addition, *Xanthobacter* species and another close relative, *Methylobacterium multivorans*, exhibit methylophily, another physiological feature shared with *Ancylobacter* strains.

DNA–DNA similarity studies of *A. aquaticus* strains B, H, M, M6, and W with the type strain show a much lower genetic relatedness (28–45%) than expected of strains of the same species, even though their mol% G + C values are very close (Konopka et al., 1976). These data indicate that the genus contains at least one other, as yet unnamed, species.

The finding that many strains of *Ancylobacter aquaticus* are facultative methylophils with a Calvin cycle and the report that

several strains can grow as hydrogen autotrophs indicate these bacteria share some properties with methylophils and chemolithotrophic bacteria.

“Renobacter vacuolatum” Nikitin 1971, a new genus and species proposed for nonmotile, gas-vacuolated bacteria, is now regarded as a member of the genus *Ancylobacter* (Namsaraev, 1973; J. Larkin, personal communication).

The original strain described by Ørskov as the type strain of *A. aquaticus* has been lost (Ørskov, 1953). Subsequently, new strains have been isolated by Ørskov (1953), and Larkin and Borrall (1979) have proposed that one of these strains (ATCC 25396) be the neotype strain.

List of species of the genus *Ancylobacter*

1. ***Ancylobacter aquaticus*** (Ørskov 1928) Raj 1983, 297^{VP} (*Micrococcus aquaticus* Ørskov 1928, 128.)
a. qua' ti. cus. L. adj. *aquaticus* living in water.

The characteristics are as described for the genus. Occur in soil and freshwater.

The mol% G + C of the DNA is: 66–69 (Bd).

Type strain: Ørskov, ATCC 25396.

GenBank accession number (16S rRNA): M62790.

Genus IV. ***Angulomicrobium*** Vasilyeva, Lafitskaya and Namsaraev 1986, 354^{VP} (Effective publication: Vasilyeva, Lafitskaya and Namsaraev 1979, 1037)

LEANA V. VASILYEVA

An' gu. lo. mi. cro' bi. um. M.L. fem. n. *angularis* angular; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. neut. n. *Angulomicrobium* angular microbe.

Unicellular bacterium, having polygonal cells with radial symmetry, ranging in dimensions from 1.1–1.5 µm. The shape of the cells is tetrahedral or mushroom-like. Flat, triangular bacteria are provisionally included within the genus.

Cells divide by budding. Buds are produced on the tetrahedron directly on the conical point of elongation of the mother cell, with a short tube connecting two cells. **Gram negative. Non-motile.** Cells lack prosthecae, lamellated membranous structures, and gas vacuoles.

Obligately aerobic, nonfermentative, **chemoorganotrophic**. A variety of organic acids, monosaccharides, and amino acids are utilized. Methanol and formate can serve as energy sources only in the presence of yeast extract. Methane and hydrogen are not utilized. **Catalase and oxidase positive.** Monotypic.

The mol% G + C of the DNA is: 64.3–68.

Type species: ***Angulomicrobium tetraedrale*** Vasilyeva, Lafitskaya and Namsaraev 1986, 354 (Effective publication: Vasilyeva, Lafitskaya and Namsaraev 1979, 1037.)

FURTHER DESCRIPTIVE INFORMATION

Budding bacteria that resemble a “mushroom” during some stages of the growth cycle have been isolated from aquatic environments (Whittenbury and Nicoll, 1971; Namsaraev and Zavarzin, 1972; Lafitskaya and Vasilyeva, 1976; Stanley et al., 1976). Slide cultures indicate that the bacteria reproduce by a budding process. Newly divided organisms are rounded on one side, while the area where cell separation occurs appears conical. This conical section elongates to form a tube, and at this stage, the cell outline resembles a mushroom. The growing tube then enlarges. Just before cell division, the mother and daughter cells are of equal size. The region between the mother and daughter cells constricts, and the two cells separate. Both mother and daughter

cells synchronously produce lateral buds at the point of separation (see Fig. BXII.α.198).

The same cycle is observed for the flat, triangular bacteria. After separation, growth begins by elongation of the apex of the triangle at the point of division, and new buds are formed synchronously by the mother and daughter cells (Vasilyeva et al., 1979).

The cell wall structure is typical of Gram-negative bacteria, but the flat triangular form, in contrast to the form of the type strains, has no visible rigid layer in its cell wall. No membranous structures are observed, except for the small loops of membranes close to the cell wall (Fig. BXII.α.199).

All strains are capable of growth in the mineral medium with glucose or a variety of other carbohydrates as sole sources of energy and organic carbon. None of the strains grow anaerobically, with or without nitrate or sulfate. Sugars are used via the hexose–monophosphate and Entner–Doudoroff pathways.

Strain Z-2821 can grow with methanol or formate as a sole energy source, but in this case a limited amount of yeast extract should be added (Namsaraev and Zavarzin, 1974). Acetate enhances methylophily growth. Hydrolytic activity is absent (Lafitskaya and Vasilyeva, 1976).

DNA hybridization reassociation shows no relation of the mushroom-shaped bacteria to *Hyphomicrobium*, *Rhodopseudomonas*, *Ancalomicrobium*, or *Prosthecomicrobium* (Stanley et al., 1976).

The flat, triangular strain 1109 differs from the type strains and might be representative of another taxon. In particular, the shape of its flat cells suggests it may be closer to the prosthecobacteria *Stella* and *Labrys*. However, no further work has been done, and it is premature to establish a new species until additional isolates have been characterized.

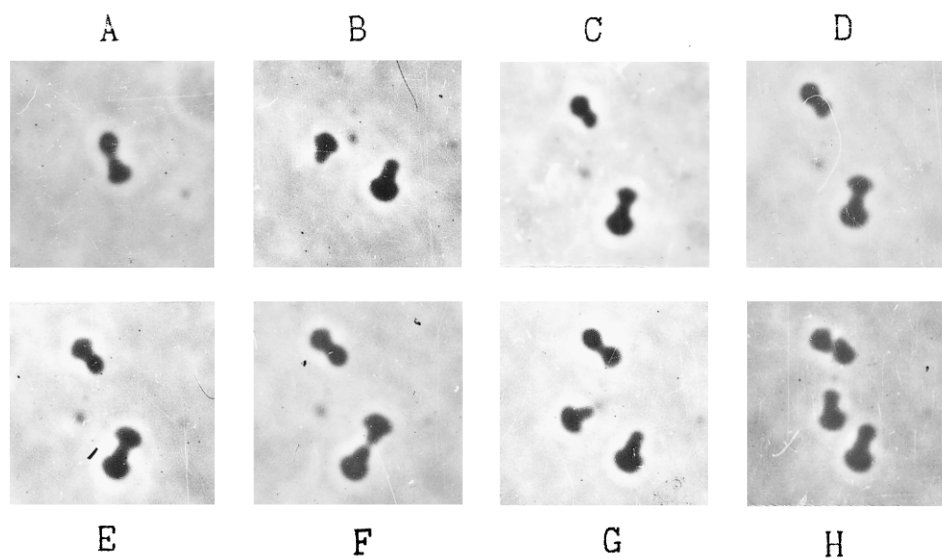


FIGURE BXII.α.198. *A. tetradrale*. Sequential series of phase photomicrographs illustrating the growth cycle of the type strain. Time in hours: A, 0; B, 0.5; C, 1; D, 2; E, 2.5; F, 3.5; G, 4; and H, 4.5. Bar = 5 μ m. (Reproduced with permission from L.V. Vasilyeva et al., *Microbiologiya* 48: 1033–1039, 1979.)



FIGURE BXII.α.199. Thin section of *Angulomicrobium*. A, type strain AUCM B-1335. Bar = 0.5 μ m. B, flat triangular AUCM B-1336. Bar = 0.5 μ m.

ENRICHMENT AND ISOLATION PROCEDURES

No selective procedure for enrichment is known. Strains have been isolated as colonies on a mineral base medium supplemented by low concentrations of organic substances, e.g., glucose. Numerous colonies are examined in wet mounts by phase-

contrast microscopy, and those containing cells with unusual morphology isolated. The type strain was isolated as a satellite in a methane-oxidizing community from a swamp (Namsaraev and Zavarzin, 1972). Another strain has been isolated from freshwater (Whittenbury and Nicoll, 1971). A pulp mill aeration lagoon has been found to contain up to 10^6 cells/ml (Stanley et

al., 1976). The flat triangle was isolated from a peat bog in the Moscow River valley (Lafitskaya and Vasilyeva, 1976).

MAINTENANCE PROCEDURES

Strains can be maintained on slants in the refrigerator for at least 6 months. Potato infusion agar and mineral base medium (e.g., MMB agar, see *Ancalomicrobium*), with glucose or other sugars, are favorable growth media. Lyophilization can be used for long term preservation.

DIFFERENTIATION OF THE GENUS *ANGULOMICROBIUM* FROM OTHER GENERA

Angulomicrobium is differentiated from other genera of budding bacteria by its characteristic morphology. It differs from *Labrys* by the absence of prosthecae, and it differs from *Nitrobacter* and similar budding bacteria by the absence of lamellated membranous structures. The flat, triangular strain 1109 might be compared with the flat, dividing bacteria with radial symmetry of the genus *Stella*.

TAXONOMIC COMMENTS

Angulomicrobium represents a distinct morphological type in the oligocarbophilic "microflora of dispersal" (Zavarzin, 1970), or

among the dissimilatory microorganisms, as it is called now (Vasilyeva and Zavarzin, 1995). It utilizes either carbohydrates in low concentrations or methanol produced by incomplete oxidation of methane in methane-utilizing communities. Substrate utilization is similar for all strains, except the flat, triangular strain. It is not related to other genera of budding bacteria by DNA-DNA similarity studies. On the basis of DNA base composition (mol% G + C of the DNA = 68) the strains of *Angulomicrobium* cannot be closely related to other budding bacteria, such as *Gemmiger formicilis* (mol% G + C of the DNA = 59), *Nitrobacter* (mol% G + C of the DNA = 61–62), or *Hyphomicrobium* (mol% G + C of the DNA = 59–61); they are close to *Rhodospseudomonas palustris* (mol% G + C of the DNA = 65–66), but do not possess intracytoplasmic membranous structures (Stanley et al., 1976). DNA-DNA homology studies indicate a relatedness among all four strains of *Angulomicrobium* in the range of 40–85% (Stanley et al., 1976).

Sequencing 16S rRNA studies indicate that *Angulomicrobium tetraedrale* (strain WAL-4, Stanley et al., 1976) belongs to the *Alpha-proteobacteria* and is most closely related to *Rhodospseudomonas acidiphila* (now *Rhodoblastus acidophilus* and *R. palustris* (Vasilyeva, 1989).

List of species of the genus *Angulomicrobium*

1. ***Angulomicrobium tetraedrale*** Vasilyeva, Lafitskaya and Namsaraev 1986, 354^{VP} (Effective publication: Vasilyeva, Lafitskaya and Namsaraev 1979, 1037.)
tel'ra.ed'ra.le. Gr. pref. *tetra-* four; Gr. n. *edra* seat, face; Gr. adj. *tetraedralis* tetrahedral.

Unicellular, budding bacterium. The cells have radial symmetry; cells of the type strain have a tetrahedral form, 1.1–1.5 μm (Fig. BXII.α.200); cells of other strains (1109) have a flat form, 0.95–1.2 μm (Fig. BXII.α.201). Single or in pairs. Gram negative. Nonmotile.

Multiplication is by budding from the top of the tetrahedron (Fig. BXII.α.198) or from the top of the triangle. Mother and daughter cells are similar in form and size. Ultrastructure typical of Gram-negative bacteria, with no complex membranous system (Fig. BXII.α.199).

Colonies are white, round, and mucous with a pearly shine.

Chemoorganotroph. Carbohydrates, amino acids, organic acids, and alcohols are utilized as sole sources of carbon and energy. All strains utilize arabinose, glucose, L-histidine, L-proline, acetate, and mannitol. Some strains utilize mannose, ribose, xylose, propionate, citrate, ethanol, methanol, and methylamine; no strains utilize lactose, raffinose, pectin, glycogen, gelatin, L-tryptophan, L-arginine, L-leucine, DL-methionine, or Tween-80.

Only the flat strain 1109 utilizes sucrose, cellobiose, and melibiose, but this strain does not utilize methanol, organic acids, or glycerol. Organic acids are not produced from carbohydrates.

Sugars are used via the hexose-monophosphate and Entner-Doudoroff pathways. Type strain has enzymes of the Embden-Meyerhof pathway (Lafitskaya and Vasilyeva, 1976).

Aerobic. Catalase and oxidase positive.

Organic growth factors are not required, but they stimulate growth. Strain 1109 requires yeast extract.

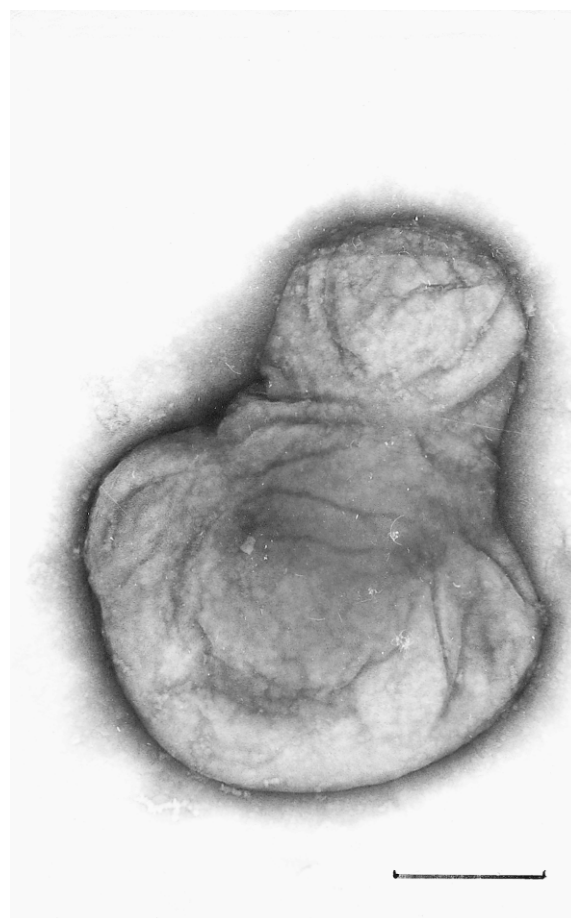


FIGURE BXII.α.200. Cell of type strain AUCM B-1335 with phosphotungstic acid. Bar = 0.5 μm . (Reproduced with permission from E.N. Michustin, *Izvestiya Akademii Nauk S.S.S.R., Seriya Biologicheskaya* 5: 719–737, 1980.)

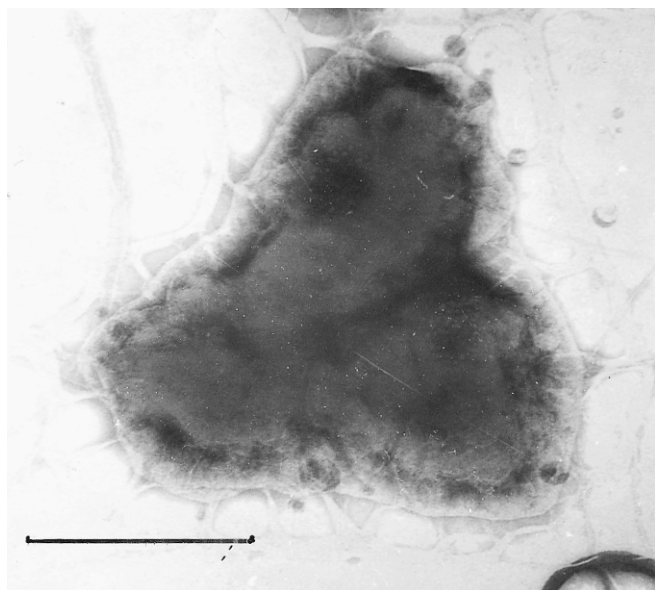


FIGURE BXII.α.201. Cell strain AUCM B-1336 with phosphotungstic acid. Bar = 0.5 μ m. (Reproduced with permission from E.N. Michustin, *Izvestiya Akademii Nauk S.S.S.R., Seriya Biologicheskaya* 5: 719–737, 1980.)

Genus V. Aquabacter Irgens, Kersters, Segers, Gillis and Staley 1993, 864^{VP} (Effective publication: Irgens, Kersters, Segers, Gillis and Staley 1991, 141)

JAMES T. STALEY

Aq'ua.bac.ter. L. n. *aqua* water; Gr. neut. n. *bacter* masc. form of Gr. neut. n. *bacterion* a rod; N.L. masc. n. *Aquabacter* aquatic rod.

Rod-shaped cells, 0.5–1.0 \times 1.5–3.0 μ m. **Unicellular.** Encapsulated. **Motile by flagella, but only under certain conditions.** No resting stages known. **Gram negative.**

Aerobic. Chemoheterotrophic. Organic acids including some amino acids used aerobically as carbon source. Sugars not known to be used. Growth occurs on mineral medium with acetate, succinate, or pyruvate as sole carbon source and inorganic ammonium compounds as sole nitrogen source, along with B-vitamins. Oxidase and catalase positive. Type strain, which is gas vacuolate, was isolated from Spirit Lake, Washington following the eruption of Mt. St. Helens in 1980.

The mol% G + C of the DNA is: 67.

Type species: ***Aquabacter spiritensis*** Irgens, Kersters, Segers, Gillis and Staley 1993, 864 (Effective publication: Irgens, Kersters, Segers, Gillis and Staley 1991, 141.)

FURTHER DESCRIPTIVE INFORMATION

The 16S rDNA sequence has not yet been determined for the type species.

This monospecific genus belongs to the *Alphaproteobacteria* based on DNA–rRNA hybridization (Irgens et al., 1991). Within that group, it is most closely related to *Azorhizobium* and *Xanthobacter*, but it differs phenotypically and phylogenetically from these two genera (Table BXII.α.167).

Aquabacter spiritensis is not motile when grown in typical broth media. However, flagellation occurs when cells are grown on a semisolid medium as described by Lara and Konopka (1987).

Gas vacuoles are produced by the type strain when grown on typical broth or solid media.

The type strain uses a limited number of carbon sources. Some

Temperature range: 15–35°C; optimal temperature: 28–30°C. Optimal pH for growth: near neutrality (6.8–7.0).

Sensitivity towards antibiotics (μ g/ml) for type strain and 1109: penicillin G, 30 and 100, respectively; chloramphenicol, 100 and 0.5; erythromycin, 10 and 30; actinomycin D, 5.0 and 8.0; and polymyxin M, 50 and 10.

Habitat: type strain isolated from a swamp near Moscow. Other related strains isolated from a freshwater and pulp mill aeration lagoon.

The mol% G + C of the DNA is: 64.5 (T_m , type strain) (68.2 according to Stanley et al., 1976); 64.3 (strain 1109).

Type strain: Strain Z-2821, AUCM B-1335, DSM 5895.

GenBank accession number (16S rRNA): AS535708.

organic acids such as lactate, pyruvate, acetate, malate, succinate, propionate, and butyrate, as well as L-glutamate and L-alanine, are used as carbon sources in complex media. However, none of the sugars or alcohols that have been tested (D-glucose, D-fructose, DL-arabinose, rhamnose, sucrose, maltose, lactose, mannitol, sorbitol, glycerol, ethanol) are used as carbon sources for growth.

ENRICHMENT AND ISOLATION PROCEDURES

The sole strain of this genus was isolated from Spirit Lake, Washington, following the eruption of Mt. St. Helens volcano in 1980. A sample collected at 5.0 m depth in the lake on 10 April 1981, a little more than a year after the eruption, was diluted to 10^{-4} and plated out on dilute peptone agar containing (per liter): Bacto Peptone, 100 mg; Hutner's salts solution (see *Ancalomicrobium*, this volume), 20 ml; agar, 15 g. After prolonged incubation at room temperature, only one colony had the chalky white appearance typical of gas vacuolate bacteria.

MAINTENANCE PROCEDURES

The organism can be maintained by lyophilization.

DIFFERENTIATION OF THE GENUS *AQUABACTER* FROM OTHER GENERA

Table BXII.α.167 shows the differences between *Aquabacter*, *Xanthobacter*, and *Azorhizobium*. *Aquabacter spiritensis* does not use the typical carbon sources used by *Azorhizobium* and *Xanthobacter* spp. In addition, although all three genera resemble one another in morphology, the type strain and species of *Aquabacter* is gas vacuolate.

TABLE BXII.α.167. Differentiation of the genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter*

Characteristics	<i>Aquabacter</i>	<i>Azorhizobium</i>	<i>Xanthobacter</i>
Motility	+ ^a	+	D
Colony color	chalky white	cream	yellow
Gas vacuole formation	+ ^b	—	—
Utilization of:			
Glucose	—	+	3/16 ^c
DL-Proline	—	19/20 ^c	3/16 ^c
Methanol, ethanol	—	—	15/16 ^c
Gluconate	—	+	12/16 ^c
L-Lysine	—	+	—

^aMotility is not ordinarily observed (see text).^bOnly one strain has been described, and it is gas vacuolate.^cFractions indicate the number of positive strains of the total number of strains tested.*List of species of the genus Aquabacter*

1. ***Aquabacter spiritensis*** Irgens, Kersters, Segers, Gillis and Staley 1993, 864^{VP} (Effective publication: Irgens, Kersters, Segers, Gillis and Staley 1991, 141.)

spi.ri.ten'sis. N.L. adj. *spiritensis* named after Spirit Lake, Washington, USA, from which the strain was isolated.

Gas vacuolate, nonflagellated rods. Typically nonmotile, however, flagella are produced under special conditions.

Obligately aerobic. Growth occurs at 25–37°C, but not at 20 or 40°C. Nitrate is reduced to nitrite. Nitrogen is not fixed. B-vitamins are required for growth on defined media. The following carbon sources are utilized: acetate, succi-

nate, pyruvate, lactate, propionate, butyrate, L-glutamate, and L-alanine.

Colonies are circular, slightly convex in elevation, with an entire margin and smooth, glistening surface. Colonies may appear translucent or chalky white if cells are gas vacuolate. Growth on older slant cultures has a rubbery texture.

The type strain was isolated from Spirit Lake, WA USA.

The mol% G + C of the DNA is: 67 (*T_m*).

Type strain: SPL-1, ATCC 43981, DSM 9035, LMG 8611.

Genus VI. *Azorhizobium* Dreyfus, Garcia and Gillis 1988, 89^{VP}

L. DAVID KUYKENDALL

A.zo.rhi.zo'bi.um. Fr. n. *azote* nitrogen; M.L. neut. n. *Rhizobium* a bacterial generic name; M.L. neut. n. *Azorhizobium* a nitrogen (using) rhizobium.

Rods 0.5–0.6 × 1.5–2.5 μm. Motile by one polar or subpolar flagellum. **Nonsporeforming. Gram negative. Peritrichous flagella are formed on solid media, and a single lateral flagellum is formed in broth.** Growth temperature, 12–43°C. Broad optimum pH, 5.5–7.8. Colonies are circular and creamy. Urease negative. **Oxidase and catalase positive. Do not denitrify. Of sugars, only glucose is oxidized.** The organic acids lactic acid and succinic acid are preferred substrates for growth, both under and not under nitrogen-fixing conditions. Growth on malonate and DL-proline. Starch is not hydrolyzed. Nitrogen-fixing root- and stem-nodulating microsymbiont of *Sesbania rostrata*. Readily **fixes nitrogen ex planta under microaerobic conditions and with nicotinic acid provided.** Can grow on nitrogen-free medium, unlike all other legume microsymbionts, of the genera *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium*, which lie in distinct families.

The mol% G + C of the DNA is: 66–68.

Type species: ***Azorhizobium caulinodans*** Dreyfus, Garcia, and Gillis 1988, 89.

FURTHER DESCRIPTIVE INFORMATION

Nitrogen-fixing stem nodules are formed on *Sesbania rostrata* by *Azorhizobium caulinodans* ORS 571^T (Fig. BXII.α.202). *Azorhizobium* Nod factors, responsible for the induction of nodule organogenesis, have been characterized by Mergaert et al., 1993. The nodulation genes, *nod*, have been studied by Goethals et al., (1989, 1990, 1992a, b), and by Geelen et al., (1993). A small rod-shaped cell of *Azorhizobium caulinodans* ORS 571^T with a single lateral flagellum is depicted in Fig. BXII.α.203.

ENRICHMENT AND ISOLATION PROCEDURES

Azorhizobia are readily isolated from stem nodules on *Sesbania rostrata*, using the same procedures described for the isolation of *Bradyrhizobium* from soybean.

MAINTENANCE PROCEDURES

Azorhizobium will grow on a chemically defined minimal medium without added nitrogen. LO medium was defined by Dreyfus et al., (1983). Storage recommendations are the same as given for *Rhizobium*.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

For verification of *Sesbania* nodulation, plant seeds are both scarified and surface-sterilized by immersion in concentrated H₂SO₄ for 15–30 min, then washed several times with sterile water and germinated on semisolid agar. Seedlings are placed in 25 × 150-mm plugged culture tubes on agar slopes of Jensen seedling agar (Vincent, 1970).

DIFFERENTIATION OF THE GENUS *AZORHIZOBIUM* FROM OTHER GENERA

Growth on a nitrogen-free medium (Dreyfus et al., 1988) clearly distinguishes *Azorhizobium* from the other, not very closely related, legume-nodulating bacteria *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*. *Azorhizobium* is distinguished from close relatives like *Xanthobacter*, *Aquabacter*, and other members of the *Hyphomicrobiaceae* by the inability of these species to form nitrogen-fixing nodules on *Sesbania*.

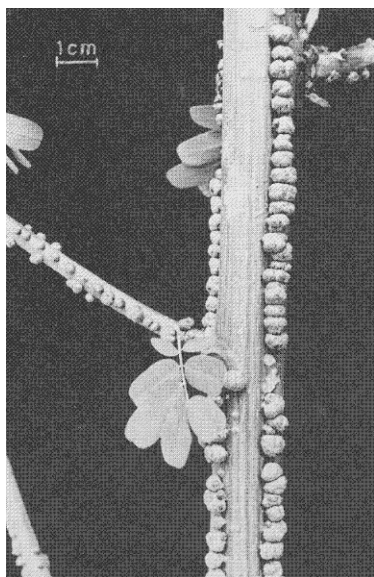


FIGURE BXII.α.202. Stem nodules formed by *Azorhizobium caulinodans* ORS 1^T on *Sesbania rostrata* (Reproduced with permission from B. Dreyfus et al., *International Journal of Systematic Bacteriology* 38: 89–98, 1988, ©International Union of Microbiological Societies.)

TAXONOMIC COMMENTS

At the time of the first edition of *Bergey's Manual of Systematic Bacteriology*, the bacteria that form stem nodules on *Sesbania rostrata* had not yet been named, *Bradyrhizobium* was still within the family *Rhizobiaceae*, and the only three *Rhizobium* species were *R. leguminosarum*, *R. meliloti*, and *R. loti*. *R. loti* is now in the separate genus *Mesorhizobium*. Since the microsymbionts of *Sesbania rostrata* (Dreyfus et al., 1983) are distinctly different from *Bradyrhizobium* and *Rhizobium*, both genotypically and phenotypically, Dreyfus et al. (1988) placed them in a new genus. FAME and 16S rRNA analysis have been used to confirm the clear separation of *Azo-*

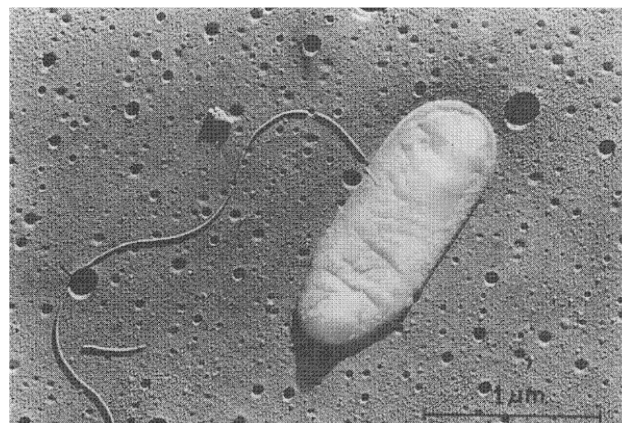


FIGURE BXII.α.203. A single lateral flagellum attached to a cell of *Azorhizobium caulinodans* ORS 1^T, as shown by negative staining and electron micrography of a diluted broth culture (Reproduced with permission from B. Dreyfus et al., *International Journal of Systematic Bacteriology* 38: 89–98, 1988, ©International Union of Microbiological Societies.)

rhizobium from both *Bradyrhizobium* and *Rhizobium* (So et al., 1994). Van Rossum et al. (1995) have also reconfirmed their distinct lineages. Because of their significant phylogenetic dissimilarity with the *Rhizobiaceae* and with the *Bradyrhizobiaceae*, it is appropriate that these organisms be in a distinct family, *Hyphomicrobiaceae*, based on 16S rRNA similarity.

Only one species of *Azorhizobium* is presently recognized. *Azorhizobium* has 98% 16S rDNA similarity with, and is thus very closely related to, *Xanthobacter* (Rainey and Wiegel, 1996) and these genera will probably be amalgamated, as might *Aquabacter*, since the latter shares approximately 97% 16S rDNA similarity with both *Azorhizobium* and *Xanthobacter*.

ACKNOWLEDGMENTS

The author is indebted to Dr. B. Dreyfus for permission to reprint the photographs.

List of species of the genus *Azorhizobium*

1. ***Azorhizobium caulinodans*** Dreyfus, Garcia, and Gillis 1988, 89.
cau.li' no.dans. M.L. n. *caulis* stem; M.L. v. *nodare* to nodulate; M.L. part. adj. *caulinodans* stem-nodulating.

The characteristics are as described for the genus. Strains effectively nodulate the roots and stems of *Sesbania rostrata*. Grow with 8% potassium nitrate present. Possess arginine and lysine decarboxylase. Do not utilize D-mannitol. Grow on azelate, maleate, adipate, pimelate, suberate, gluconate, mucate, crotonate, nicotinate, 2-ketogluconate, propionate,

butyrate, isobutyrate, valerate, isovalerate, caproate, laurate, 2-ketoglutarate, fumarate, glutarate, sebacate, DL-malate, citrate, pyruvate, aconitate, citraconitate, D-glucuronate, α-D-galacturonate, *m*-hydroxybenzoate, L-aspartate, quinate, L-α-alanine, L-lysine, L-asparagine, betaine, and sarcosine. Nicotinic acid must be supplied as a vitamin supplement for growth under nitrogen-limiting conditions.

The mol% G + C of the DNA is: 66–68 (*T_m*).

Type strain: ORS 571, LMG 6465.

GenBank accession number (16S rRNA): D13948, D11342, X67221, X94200.

Genus VII. *Blastochloris* Hiraishi 1997, 218^{VP}

JOHANNES F. IMHOFF

Blas.to.chlo' ris. Gr. n. *blastos* bud shoot; Gr. adj. *chloros* green; M.L. fem. n. *Blastochloris* green bud shoot.

Cells are rod shaped to ovoid and motile by means of subpolar flagella. They exhibit polar growth, budding, and asymmetric cell division and form rosette-like cell aggregates. They are **Gram negative and belong to the Alphaproteobacteria**. Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *b* and carotenoids. Straight-chain monounsatu-

rated C_{18:1} is the predominant component of cellular fatty acids. Ubiquinones and menaquinones are present, and the lipopolysaccharides are characterized by a 2,3-diamino-2,3-deoxy-D-glucose (DAG)-containing, phosphate-free lipid A with amide-bound C_{14:0} 3OH.

Preferred growth mode is photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth may be

possible under anoxic conditions with thiosulfate or sulfide as electron donor. **Chemotrophic growth is possible** under microoxic conditions in the dark. Growth factors may be required.

Mesophilic freshwater bacteria with a preference for neutral pH.

The mol% G + C of the DNA is: 66.3–71.4.

Type species: Blastochloris viridis (Drews and Giesbrecht 1966) Hiraishi 1997, 218 (*Rhodopseudomonas viridis* Drews and Giesbrecht 1966, 261.)

FURTHER DESCRIPTIVE INFORMATION

The cell morphology of the *Blastochloris* species is characterized by asymmetric growth and cell division. Most characteristic is the formation of prosthecae and rosette-like cell aggregates as in *Rhodopseudomonas* species.

Blastochloris sulfoviridis is unable to assimilate sulfate and depends on reduced sulfur compounds (Neutzing and Trüper, 1982), whereas *Blastochloris viridis* can grow with sulfate as the sole sulfur source and assimilates sulfate via the 3'-phosphoadenosine-5'-phosphosulfate pathway (Imhoff, 1982). Reduced sulfur compounds, sulfide, and thiosulfate are used as photosynthetic electron donors by *B. sulfoviridis*, but not *B. viridis*, and oxidized to sulfate (Keppen and Gorlenko, 1975). The lipid A structure of the lipopolysaccharide of *Blastochloris viridis* and *Blastochloris sulfoviridis* (Ahamed et al., 1982; Weckesser et al., 1995) contains the unusual 2,3-diamino-2,3-deoxy-D-glucose instead of glucosamine. This sugar has also been found in other species of the *Alphaproteobacteria* such as *Rhodopseudomonas palustris* and *Nitrobacter winogradskyi*, but not *Rhodomicrobium vannielii* and *Rhodoblastus acidophilus* (Weckesser et al., 1995).

ENRICHMENT AND ISOLATION PROCEDURES

Standard procedures for the isolation of purple nonsulfur bacteria (PNSB) can be applied. A mineral salts medium, which is suitable for most PNSB, is also applicable for the cultivation of *Blastochloris* species (Imhoff, 1988; Imhoff and Trüper, 1992; see chapter Genus *Rhodospirillum* for this medium recipe). *B. viridis* and *B. sulfoviridis* can be selectively enriched with appropriate light filters, that allow only long wavelength radiation to penetrate, since both species, owing to their content of bacterio-

chlorophyll *b*, show absorption maxima above 1000 nm. The dependence on reduced sulfur compounds demands the addition of low concentrations of sulfide or cysteine into media for *B. sulfoviridis*.

MAINTENANCE PROCEDURES

Cultures of *Blastochloris* species can be preserved in liquid nitrogen or at –80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *BLASTOCHLORIS* FROM OTHER GENERA

16S rDNA sequence analysis places the genus in the *Alphaproteobacteria*. *Rhodoplanes* species are the closest relatives among phototrophic bacteria. *Blastochloris* species may well be differentiated from other PNSB based on morphological and cultural characteristics. Their green to olive-green coloration, together with the characteristic long wavelength absorption maximum above 1000 nm, which is due to bacteriochlorophyll *b*, clearly separates *Blastochloris* species from other phototrophic *Alphaproteobacteria*. The rod-shaped cells, their asymmetric mode of growth and cell division, the formation of rosette-like aggregates, the lamellar structure of internal photosynthetic membranes lying parallel to and underlying the cytoplasmic membrane, as well as a number of chemotaxonomic characteristics and the sequences of 16S rDNA, differentiate *Blastochloris* species from other genera of the purple nonsulfur bacteria (see Tables 3 and 4 [pp. 125–127] as well as Fig. 2 [p. 128] of the introductory chapter “Anoxygenic Phototrophic Purple Bacteria” in Volume 2, Part A).

TAXONOMIC COMMENTS

Both species of this genus have been previously assigned to the genus *Rhodopseudomonas*, because of significant similarities in cell morphology and cell division to *Rhodopseudomonas palustris*. Significant differences in 16S rDNA sequences with respect to other PNSB have given reason to reconsider the taxonomic position of these bacteria and reevaluate phenotypic differences from the type species of *Rhodopseudomonas*, leading to their reassignment to the new genus *Blastochloris* as *Blastochloris viridis* and *Blastochloris sulfoviridis* (Kawasaki et al., 1993b; Hiraishi, 1997).

DIFFERENTIATION OF THE SPECIES OF THE GENUS *BLASTOCHLORIS*

Major differentiating properties between *Blastochloris* species and between *Blastochloris*, *Rhodomicrobium vannielii*, and *Rhodoplanes* species are shown in Tables BXII.α.168 and BXII.α.169.

List of species of the genus *Blastochloris*

1. ***Blastochloris viridis*** (Drews and Giesbrecht 1966) Hiraishi 1997, 218^{VP} (*Rhodopseudomonas viridis* Drews and Giesbrecht 1966, 261.)
vi'ri.dis. L. adj. *viridis* green.

Cells are rod-shaped to ovoid, 0.6–0.9 × 1.2–2.0 μm. The mother cell produces a slender prostheca, 1.5–2.0 times the length of the original cell, at the pole opposite that bearing the flagella. The end of the prostheca swells, and the daughter cell grows, producing a dumbbell-shaped organism, just like *Rhodopseudomonas palustris* (see Fig. BXII.α.187 in the chapter describing the species *Rhodopseudomonas palustris*). Asymmetric division then takes place. In young cultures, swarmer cells motile by means of subpolar flagella are frequent. The formation of rosettes and clusters in which the individual cells are attached to each other at their flagellated poles are characteristic in older

cultures. Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane. The color of photosynthetic cultures is first yellowish green, then green to olive green. Aerobic cultures are colorless to light yellowish green. *In vivo* absorption spectra show characteristic maxima at 400, 420, 451, 483, 604, 835, and 1020 nm. Photosynthetic pigments are bacteriochlorophyll *b* (esterified with phytol), with 1,2-dihydroneurosporene and 1,2-dihydrolycopene as major carotenoids.

Cells grow photoheterotrophically under anoxic conditions with organic carbon sources. Photoautotrophic growth has not been demonstrated. Chemotrophic growth is possible under microoxic conditions in the dark. Carbon sources used are acetate, pyruvate, malate, and succinate. Poor growth occurs in the presence of ethanol, glutamate, peptone, glucose, and xylose. Longer chain fatty acids in-

TABLE BXII.α.168. Differential characteristics of the anoxygenic phototrophic purple bacteria belonging to the family *Hyphomicrobiaceae* of the order *Rhizobiales*: genera *Blastochloris*, *Rhodomicrobium*, and *Rhodoplanes*^a

Characteristic	<i>Blastochloris viridis</i>	<i>Blastochloris sulfovirens</i>	<i>Rhodomicrobium vannielii</i>	<i>Rhodoplanes roseus</i>	<i>Rhodoplanes elegans</i>
Cell diameter (μm)	0.6–0.9	0.5–0.9	1.0–1.2	1.0	0.8–1.0
Type of budding	Tube	Sessile	Tube	Sessile	Tube
Rosette formation	+		Complex aggregates	–	+
Internal membrane system	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae
Motility	+	+	+	+	+
Color of cultures	Green to olive-green	Olive-green	Orange-brown to red	Pink	Pink
Bacteriochlorophyll	<i>b</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>a</i>
Salt requirement	None	None	None	None	None
Optimal pH	6.5–7.0	7.0	6.0	7.0–7.5	7.0
Optimal temperature	25–30	28–30	30	30	30–35
Sulfate assimilation	+	–	+	nd	nd
Aerobic dark growth	(+)	(+)	+	+	+
Denitrification	–	–	nd	+	+
Fermentation of fructose	nd	nd	nd	–	–
Photoautotrophic growth with	–	Thiosulfate, sulfide	H ₂ , sulfide	Thiosulfate	Thiosulfate
Growth factors	Biotin, <i>p</i> -amino-benzoic acid	Biotin, <i>p</i> -amino-benzoic acid, pyridoxin	None	Niacin	Thiamine, <i>p</i> -amino-benzoic acid
<i>Utilization of:</i>					
Benzoate	–	–	–	–	–
Citrate	–	–	–	+	+
Formate	–	–	+ / –	–	–
Glucose	(+)	+	–	–	–
Tartrate	–	–	–	+	+
Sulfide	–	+	+	–	–
Thiosulfate	–	+	+	+	+
Mol % G + C of the DNA	66.3–71.4 (Bd)	67.8–68.4 (CA)	61.8–63.8 (Bd)	66.8 (HPLC)	69.6–69.7 (HPLC)
Cytochrome <i>c</i> ₂ size	Small	nd	Small	nd	nd
Major quinones	Q-9, MK-9	Q-8/10, MK-7/8	Q-10, RQ-10	Q-10, RQ-10	Q-10, RQ-10
<i>Major fatty acids</i>					
C _{14:0}	0.5	2.5	2.4		
C _{16:0}	8.4	8.6	3.7		
C _{16:1}	5.5	9.2	0.6		
C _{18:0}	2.2	1.7	3.6		
C _{18:1}	74.6	76.5	85.6		

^a+, positive in most strains; –, negative in most strains; +/–, variable in different strains; nd, not determined; (+), weak growth or microaerobic growth only; (APS), via adenosine-5'-phosphosulfate; (PAPS), via 3'-phosphoadenosine-5'-phosphosulfate; (biotin) biotin is required by some strains; Q-10, ubiquinone 10; MK-10, menaquinone 10; RQ-10, rhodoquinone 10. Bd, buoyant density; CA, chemical analysis.

hibit growth. Sulfide, thiosulfate, and hydrogen cannot be used as electron donors. Sulfate can be used as the sole sulfur source. Ammonia and dinitrogen are used as nitrogen sources; nitrate is not used. Most strains require biotin and *p*-aminobenzoate as growth factors; some strains require more growth factors and others none.

Mesophilic freshwater bacterium with optimal growth at 25–30°C and pH 6.5–7.0.

Ubiquinones and menaquinones with 9 isoprene (Q-9 and MK-9) units are present.

The mol % G + C of the DNA is: 66.3–71.4 (Bd).

Type strain: F, ATCC 19567, DSM 133.

GenBank accession number (16S rRNA): D25314.

- 2. *Blastochloris sulfovirens*** (Keppen and Gorlenko 1975) Hiraiishi 1997, 218^{VP} (*Rhodopseudomonas sulfovirens* Keppen and Gorlenko 1975, 258.)

sul.fo.vi'ri.dis. L. n. *sulfur* sulfur; L. adj. *viridis* green; M.L. adj. *sulfovirens* green and with sulfur.

Cells are rod-shaped to ovoid and form sessile buds and rosettes. Swarmer cells are motile by means of subpolar flagella. If cells age, they become immotile and encapsulated by slime. Internal photosynthetic membranes are pres-

ent as lamellae underlying and parallel to the cytoplasmic membrane. Color of cell suspensions is olive-green, sometimes with a brownish tinge. Photosynthetic pigments are bacteriochlorophyll *b* and carotenoids.

Growth is possible under anoxic conditions in the light or under microoxic conditions in the dark. Organic carbon sources, thiosulfate, and sulfide are used as photosynthetic electron donors. Sulfide and thiosulfate are oxidized to sulfate. Best growth occurs with glucose, fructose, maltose, sucrose, glycerol, propanol, fumarate, and malate as organic carbon sources. Cystine, cysteine, sulfide, and thiosulfate are used as sulfur sources, but sulfate is not used. Ammonia and casein hydrolysate are used as nitrogen sources, while arginine, glycine, alanine, asparagine, and urea inhibit growth. Yeast extract or biotin, pyridoxine, and *p*-aminobenzoate are required as growth factors.

Mesophilic freshwater bacterium with optimal growth at 28–30°C and pH 7.0.

The mol % G + C of the DNA is: 67.8–68.4 (chemical analysis).

Type strain: Pl, DSM 729.

GenBank accession number (16S rRNA): D86514.

TABLE BXII.α.169. Growth substrates of the anoxygenic phototrophic purple bacteria belonging to the family *Hyphomicrobiaceae* of the order *Rhizobiales*, *Blastochloris*, *Rhodomicrobium*, and *Rhodoplanes*^a

Source/donor	<i>Blastochloris viridis</i>	<i>Blastochloris sulfoviridis</i>	<i>Rhodomicrobium vannielii</i>	<i>Rhodoplanes roseus</i>	<i>Rhodoplanes elegans</i>
<i>Carbon source</i>					
Acetate	+	+	+	+	+
Aspartate	nd	nd	—	—	—
Benzoate	—	—	—	—	—
Butyrate	—	+	+	+	+
Caproate	—	nd	+	—	+
Caprylate	nd	nd	+	—	+ / —
Citrate	—	—	—	+	+
Ethanol	+	+	+	—	—
Formate	—	—	+ / —	—	—
D-Fructose	—	+	—	—	—
Fumarate	+	+	+	+	+
D-Glucose	+ / —	+	—	—	—
Glutamate	+	nd	—	—	—
Glycerol	—	+	+ / —	—	—
Glycolate	nd	nd	—	—	—
Lactate	+ / —	+	+	+	+
Malate	+	+	+	+	+
Malonate	—	—	+	—	—
Mannitol	+ / —	—	—	—	—
Methanol	—	—	+ / —	—	—
Propanol	+ / —	+	+	—	—
Propionate	—	—	+	+	+
Pyruvate	+	—	+	+	+
Sorbitol	+	+	—	—	—
Succinate	+	+	+	+	+
Tartrate	+ / —	—	—	+	+
Valerate	—	—	+	+	+
<i>Electron donor:</i>					
Sulfide	—	+	+	—	—
Thiosulfate	—	+	—	+	+

^aSymbols: +, positive in most strains; —, negative in most strains; + / — variable in different strains; nd, not determined.

Genus VIII. *Devosia* Nakagawa, Sakane and Yokota 1996, 20^{VP}

YASUYOSHI NAKAGAWA, TAKESHI SAKANE AND AKIRA YOKOTA

De.vos'i.a. M.L. dim. ending *-ia* M.L. fem. n. *Devosia* honoring Paul De Vos, a Belgian microbiologist, for his contributions to the taxonomy of pseudomonads.

Rods, 0.4–0.8 × 2.0–8.0 µm. **Motile by means of several polar flagella.** Endospores are not formed. Gram negative. **Aerobic. Oxidase and catalase positive. The major respiratory quinone is ubiquinone-10. The major cellular hydroxy fatty acids are 3-hydroxytetracosenoic acid and 3-hydroxyhexacosenoic acid. 3-Hydroxy fatty acids that are shorter than 3-hydroxyoctadecanoic acid are absent.** Phylogenetically related to members of the *Alpha-proteobacteria*.

The mol% G + C of the DNA is: 61.4.

Type species: *Devosia riboflavina* Nakagawa, Sakane and Yokota 1996, 20.

FURTHER DESCRIPTIVE INFORMATION

The following description pertains to the type strain of the type species. Cells grow vigorously on nutrient agar at 30°C. The quinone type is ubiquinone-10 (Q-10), which suggests that the organism belongs to *Alphaproteobacteria*. 2-Hydroxy (2-OH) fatty acids are not detected by thin-layer chromatography analysis. The cells contain 3-hydroxytetracosenoic acid (C_{24:1} 3OH) and 3-hydroxyhexacosenoic acid (C_{26:1} 3OH) as the major 3-OH fatty acids, and 3-hydroxyoctadecanoic acid (C_{18:0} 3OH), 3-hydroxylcosanoic acid (C_{20:0} 3OH), 3-hydroxylcosenoic acid (C_{20:1} 3OH), 3-hydroxy-

docosanoic acid (C_{22:0} 3OH), and 3-hydroxydocosenoic acid (C_{22:1} 3OH) as minor components. The long-chain acids C_{24:1} 3OH, C_{26:1} 3OH, and C_{22:1} 3OH, as well as octadecanoic acid (C_{18:0}), octadecenoic acid (C_{18:1}), and hexadecanoic acid (C_{16:0}) as fatty acid components and glucose, galactose, mannose, and glucosamine as sugar components, exist in the lipopolysaccharides (LPS) purified from the cells by extraction with phenol–chloroform–petroleum ether. Thus, the long-chain 3-OH fatty acids are components of the LPS molecule, which is an important outer membrane component. No other bacteria that contain C_{24:1} 3OH and C_{26:1} 3OH as major components have been described.

ENRICHMENT AND ISOLATION PROCEDURES

Devosia riboflavina was originally isolated from riboflavin-rich soil (Foster, 1944). The procedures for this enrichment and isolation of the strain have also been described by Foster (1944). Soil is added to a solution of 0.1% riboflavin with small amounts of K₂HPO₄ and MgSO₄·7H₂O, then incubated at room temperature. After the riboflavin has disappeared, as indicated by the loss of the orange color, the culture is streaked onto solid medium that contains same components.

MAINTENANCE PROCEDURES

Cultures can be preserved by liquid drying (L-drying). For L-drying, cells grown on nutrient agar for 48 h at 30°C are suspended in the protective medium SM1 (Sakane et al., 1996), which contains 30 g of sodium L(+)-glutamate monohydrate, 15 g of ribitol, 5 g of L-cysteine hydrochloride monohydrate in 1 liter of 0.1 M potassium phosphate buffer (pH 7.0), and dispensed in ampules. The suspension is then vacuum-dried from the liquid state without freezing and stored at 4°C. The results of an accelerated storage test suggest that cells should survive more than 20 years (Sakane and Kuroshima, 1997). Cultures may be also preserved by freezing at -80°C or -196°C. For freezing, cells are suspended in nutrient broth (Difco) containing 10% glycerol or 7% DMSO as a cryoprotective agent.

DIFFERENTIATION OF THE GENUS *DEVOSIA* FROM OTHER GENERA

Table BXII.α.170 lists properties that are useful for distinguishing the genus *Devosia*. The genus *Devosia* can be differentiated from morphologically similar genera belonging to the *Alphaproteobacteria* by certain phenotypic and chemotaxonomic characteristics and 16S rRNA sequence signatures (Table BXII.α.171). The absence of 3-OH fatty acids that are shorter than C_{18:0 3OH} and the presence of C_{24:1 3OH} and C_{26:1 3OH} as the major 3-OH fatty acids in the cells are the key differential characteristics.

TAXONOMIC COMMENTS

Phylogenetic analysis derived from 16S rRNA sequences shows that the genus *Devosia* occupies a distinct position in the *Alphaproteobacteria* (Fig. BXII.α.204). The phylogenetic independence

TABLE BXII.α.170. Some characteristics differentiating the genus *Devosia* from other morphologically similar genera of the *Alphaproteobacteria* and the genus *Pseudomonas*^a

Characteristic	<i>Devosia</i>	<i>Acetobacter</i>	<i>Acidiphilium</i>	<i>Acidomonas</i>	<i>Agrobacterium</i>	<i>Bradyrhizobium</i>	<i>Brevundimonas</i>	<i>Gluconobacter</i>	<i>Methylobacterium</i>	<i>Mycoplana</i>	<i>Rhizobium</i>	<i>Rhizomonas</i>	<i>Pseudomonas</i>	<i>Sphingomonas</i>
<i>Flagella:</i>														
Lateral		+	+	+	+				+	+	+	+		
Polar	+		+			+	+	+	+		+	+	+	+
Oxidase	+	-	D	+	D	nd	+	-	D	+	nd	+	D	nd
Catalase	+	+	nd	+	+	nd	+	nd	+	nd	nd	+	+	+
<i>Major hydroxy fatty acids:</i> ^b														
2-OH:														
C _{12:0}													+	
C _{14:0}				+				+				+		+
C _{16:0}		+		+										
(C _{16:0})			+											
3-OH:														
C _{10:0}													+	
C _{12:0}						+	+			+			+	
C _{14:0}			+	+	+	+			+		+			
(C _{14:0})										+				
C _{16:0}		+		+				+						
(C _{18:0})			+											
C _{24:1}	+													
C _{26:1}	+													
(C _{19:0 cyclo})								+						
(C _{13:0 iso})											+			
Quinone ^c	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-9	Q-10
Mol% G + C of DNA	61.4	51-65	62-70	63-65	55-63	61-65	65-68	56-64	60-70	63-68	59-64	58-65	59-68	59-68

^aSymbols: +, positive; -, negative; D, different among species and/or strains; nd, not determined.

^bParentheses indicate major hydroxy fatty acids in some species.

^cQ-9, ubiquinone 9; Q-10, ubiquinone 10.

TABLE BXII.α.171. 16S rRNA sequence signatures that distinguish the genus *Devosia* from other genera of *Alphaproteobacteria*

Position of base or base pair ^a	Base(s) in:								
	<i>Devosia</i>	<i>Rhizobiaceae</i>	<i>Erythrobacter/Sphingobacterium</i> group	<i>Azospirillum/Magnetospirillum</i> group	<i>Bradyrhizobium/Methylobacterium</i> group	<i>Brevundimonas/Caulobacter</i> group	<i>Paracoccus/Rhodobacter/Roseobacter</i> group	<i>Ehrlichia/Rickettsia</i> group	<i>Acetobacter/Gluconobacter</i> group
155:166	G:C	C:G	G/C:C/G	U:G	C:G	C:G	C/U:G/A	U/A:A/U	C/U:G
240:286	A:U	U:A	U:A	C:G	C:G	C:G	U:A	C/U:G/A	U:A
445:489	A:U	G:C	G:C	A/G:U/C	G:C	G:C	G:C	G:C/U	G:C
681:709	U:A	G:C	G:C	G/C:C/G	G:C	G:C	G/A:C/U	U:A	C:G
694	G	A	A	A	A	A	A	A	A
1419:1481	A:U	G:C	G:C	G:C	G:C	G:C	G/A:C/U	G:C/U	G:C

^a*Escherichia coli* numbering system.

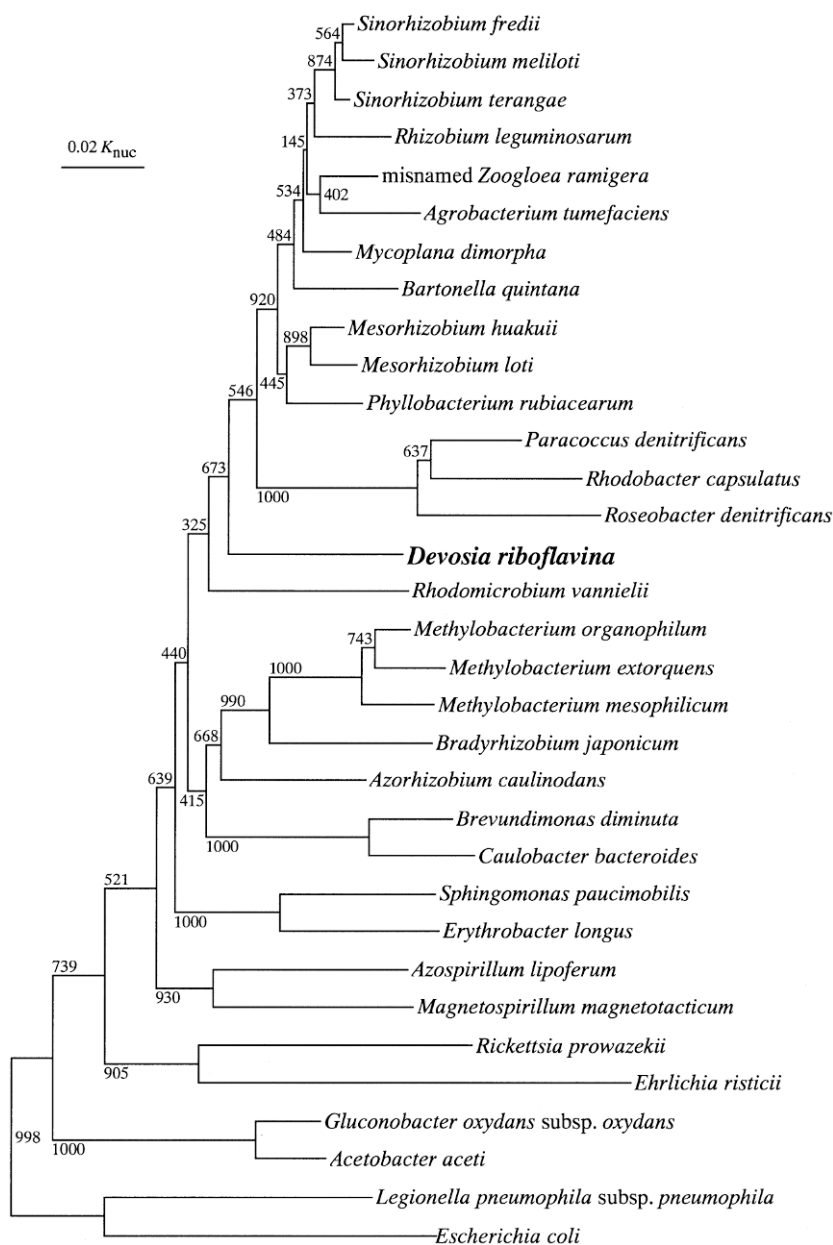


FIGURE BXII.α.204. Phylogenetic tree derived from 16S rRNA sequences of the *Alphaproteobacteria*. Clustal W ver. 1.7 (Thompson et al., 1994) was used to generate K_{nuc} values (Kimura, 1980) and to reconstruct a phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987). Scale bar = K_{nuc} values. The lengths of the vertical lines are not significant. The numbers of the branches refer to the confidence limits estimated by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. The total number of nucleotides compared with 1143 after elimination of the positions at which secondary structures varied in the strains (Nakagawa et al., 1996) and all sites which were not determined in any sequences. *Escherichia coli* and *Legionella pneumophila* subsp. *pneumophila* were used as the outgroup. The accession numbers for nucleotide sequences are as follows: *Acetobacter aceti*, X74066; *Agrobacterium tumefaciens*, M11223; *Azorhizobium caulinodans*, D11342; *Azospirillum lipoferum*, M59601; *Bartonella quintana*, M11927; *Bradyrhizobium japonicum*, D11345; *Brevundimonas diminuta*, M59064; *Caulobacter bacteroides* (This species has recently been transferred to the genus *Brevundimonas* as *B. bacteroides*), M83796; *Devosia riboflavina*, D49423; *Ehrlichia risticii*, M21290; *Erythrobacter longus*, M96744; *Escherichia coli*, J01695; *Gluconobacter oxydans* subsp. *oxydans*, X73820; *Legionella pneumophila* subsp. *pneumophila*, M59157; *Magnetospirillum magnetotacticum*, M58171; *Mesorhizobium huakuii*, D13431; *Mesorhizobium loti*, D14514; *Methylobacterium extorquens*, D32224; *Methylobacterium mesophilicum*, D32225; *Methylobacterium organophilum*, D32226; *Mycoplasma dimorpha*, D12786; *Paracoccus denitrificans*, X69159; *Phyllobacterium rubiacearum*, D12790; *Rickettsia prowazekii*, M21789; *Rhizobium leguminosarum*, D12782; *Rhodobacter capsulatus*, D16428; *Rhodococcus vannielii*, M34127; *Roseobacter denitrificans*, M59063; *Sinorhizobium fredii*, D14516; *Sinorhizobium meliloti*, D01265; *Sinorhizobium teranga*, X68387; *Sphingomonas paucimobilis*, D16144; misnamed *Zoogloea ramigera*, X74915.

of the genus *Devosia* is also reflected by the 16S rRNA signature sequences (see above).

16S rRNA cataloging (Woese et al., 1984a) and DNA–rRNA hybridization (De Vos and De Ley, 1983; De Vos et al., 1985a, 1989) have revealed that members of the genus *Pseudomonas* belong to the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. The level of heterogeneity in the genus *Pseudomonas* has been reduced by transferring various pseudomonads belonging to Palleroni’s rRNA groups (Palleroni, 1984)—other than group I—to other existing new genera (Tamaoka et al., 1987; Willems et al., 1989, 1990; Yabuuchi et al., 1990a, 1992; Segers et al., 1994).

The organism presently named *Devosia riboflavina* is one of the misnamed pseudomonads. It was originally named “*Pseudomonas riboflavina*” Foster 1944, 30 and described as a soil bacterium that oxidized riboflavin to lumichrome. In the eighth edition of the *Determinative Manual*, “*P. riboflavina*” was treated as a *species incertae sedis* because it is not motile, even though the original description indicated that it was motile (Doudoroff and Palleroni, 1974). DNA–rRNA hybridization studies (De Vos et al.,

1989; Segers et al., 1994) have shown that “*P. riboflavina*” belongs to rRNA superfamily IV (*Alphaproteobacteria*), but its precise position in the *Alphaproteobacteria* remained unknown until its 16S rRNA sequence was determined. The results of a phylogenetic analysis based on 16S rRNA sequence data (Nakagawa et al., 1996) have placed “*P. riboflavina*” IFO 13584^T in an independent position in the *Alphaproteobacteria*. The similarity rank analysis included in the Ribosomal Database Project (Maidak et al., 1997) suggests that the phylogenetic neighbors of the genus *Devosia* are members of the *Rhizobiaceae*, with which it exhibits 16S rRNA similarity values ranging from 91.9 to 93.6%.

Unfortunately, only one strain of the single species is available. Other strains belonging to the genus should be isolated and investigated in the future.

FURTHER READING

Foster, J.W. 1944. Microbiological aspects of riboflavin. I. Introduction. II. Bacterial oxidation of riboflavin to lumichrome. J. Bacteriol. 47: 27–41.

List of species of the genus *Devosia*

- 1. ***Devosia riboflavina*** Nakagawa, Sakane and Yokota 1996, 20^{VP}
ri.bo.fla'vi.na. M.L. fem. adj. *riboflavina* referring to the ability of the organism to oxidize riboflavin.

The characteristics are as described for the genus and listed in Tables BXII.α.170, BXII.α.171, and BXII.α.172. In addition, colonies are circular with entire or slightly undulate margins and cream colored. Urease positive. Organic nitrogenous substances, such as amino acids, are required for growth. Glycine, urea, and ammonium chloride cannot be substituted for organic material as nitrogen sources in media containing riboflavin as the sole energy source. Acid is produced from D-arabinose. Neither acid nor gas are produced from D-galactose, D-glucose, inositol, lactose, D-fructose, maltose, mannitol, sucrose, D-xylose, and ethanol. Esculin is hydrolyzed. Gelatin and starch are not hydrolyzed. Nitrate is slightly reduced to nitrite. Indole is not produced. Vigorous growth occurs on nutrient agar at 28°C. As determined by API 20NE tests, the following substrates are assimilated by the type strain: D-arabinose, glucose, maltose, D-mannitol, D-mannose, and N-acetyl-D-glucosamine. The following substrates are not assimilated by the type strain, as determined by API 20NE tests: adipic acid, n-capric acid, DL-malic acid, phenyl acetate, potassium gluconate, and sodium citrate. β-Galactosidase and β-glucosidase activities are present in the type strain, as determined by API 20NE tests. Arginine dihydrolase activity is not present in the type strain, as determined by API 20NE tests.

The mol% G + C of the DNA is: 61.4 (HPLC).
Type strain: 4R3337, ATCC 9526, DSM 7230, IFO 13584.
GenBank accession number (16S rRNA): D49423.

TABLE BXII.α.172. Phenotypic characteristics of <i>Devosia riboflavina</i> IFO 13584 ^a	
Test	Result
Color of colonies	Cream
Morphology of cells	Rods
Gram stain reaction	—
Motility	+
Spore formation	—
Oxidase	+
Catalase	+
Urease	+
Nitrate reduction	w
Hydrolysis of:	
Gelatin	—
Starch	—
Acid production from:	
D-Arabinose	+
D-Galactose	—
D-Glucose	—
Inositol	—
Lactose	—
D-Fructose	—
Maltose	—
Mannitol	—
Sucrose	—
D-Xylose	—
Decarboxylation of:	
L-Alanine	—
L-Lysine	—
L-Ornithine	—
Oxidation of riboflavin	+

^aSymbols: +, positive; —, negative; w, weakly positive.

Genus IX. Dichotomicrobium Hirsch and Hoffman 1989b, 495^{VP} (Effective publication: Hirsch and Hoffman 1989a, 300)

PETER HIRSCH

Di.cho' to.mi.cro.bi.um. Gr. adj. *dichotomus* divided, forked; Gr. adj. *micrus* small; Gr. n. *bios* life; M.L. neut. n. *Dichotomicrobium* a forked microbe.

Tetrahedral to spherical cells, 0.8–1.8 × 0.8–2.0 μm, with up to four prosthecae (hyphae) of 0.2–0.3 μm width and varying length. Cells and hyphae may be covered with short, rigid, bent pili. **Hyphae and mother cells may branch (fork) dichotomously.** Multiplication by terminal bud formation on hyphae or by intercalary budding. Propagation cells initially spherical or pear-shaped, later tetrahedral and never motile. Gram negative, do not form spores. **Poly-β-hydroxybutyrate (PHBA) granules may be produced, even within the hyphae.** Aerobic, moderately thermophilic and halophilic, require yeast extract for growth. Carbon sources are acetate, malate, succinate, and 2-oxoglutarate. Utilize organic nitrogen sources, such as amino acids or yeast extract; ammonium, nitrite, nitrate, molecular nitrogen, and urea do not serve as nitrogen sources. **Oxidase, catalase, and peroxidase positive.** Do not grow anaerobically. Occur in saline ponds and lakes with temperatures above 20°C.

The mol% G + C of the DNA is: 62–64.

Type species: ***Dichotomicrobium thermohalophilum* Hirsch and Hoffmann 1989b, 495 (Effective publication: Hirsch and Hoffmann 1989a, 300.)**

FURTHER DESCRIPTIVE INFORMATION

Some information on *Dichotomicrobium* isolates was initially published with the strain designation “genus D” (Gebers et al., 1985; Kölbel-Boelke et al., 1985; Roggentin and Hirsch, 1989; Sittig and Hirsch, 1992) or as “*Dichotomicrobium*” sp. (Stackebrandt et al., 1988a). All of these studies were concerned with at least isolate IFAM 954^T, the type strain for *D. thermohalophilum*.

A phylogenetic survey of budding and prosthecate bacteria by 16S rRNA cataloguing (Fig. BXII.α.205) has shown that *Dichotomicrobium* strain IFAM 954^T belongs to the *Alphaproteobacteria*, close to *Hyphomicrobium*, *Filomicrobium*, and *Pedomicrobium* species (Stackebrandt et al., 1988a).

The general morphology and life cycle of 13 strains that have been investigated is very similar (Hirsch and Hoffmann, 1989a). Mature cells are tetrahedral, triangular, or even cubical, they may be dichotomously branched. Up to four prosthecae (hyphae) grow out from the corners (Fig. BXII.α.206). The average cell size is 0.9 × 1.1 μm, with a rather constant hyphal diameter of 0.25 μm. The hyphae may branch; nearly spherical buds are produced from the hyphal tips, and only from one at a time. The buds are separated by a cross wall upon maturation and can be released, but motility of these daughter cells has never been observed. Rarely, intercalary buds are produced by a local enlargement of a hyphal portion. Sometimes, terminal buds remain attached to the hyphae and begin to grow new hyphae with a terminal bud, thus resulting in the formation of a cell chain or even a network of cells after hyphal branching. The hyphal length is dependent on nutrient concentrations: lack of nutrients (especially yeast extract) results in longer and lesser-branched hyphae. In some strains, hyphae are covered with short, rigid, but bent pili to give the appearance of a fur (Fig. BXII.α.207).

The fine structure resembles that of other typical Gram-negative bacteria. Internal membranes are absent, except for an occasional mesosome. DNA nucleoids are found in sections of

mother cells and mature buds, but cannot be demonstrated in sections of hyphae. Mother cells and often hyphae store PHBA, and most cells contain polyphosphate granules. Hyphal cross sections reveal an exceptionally dense and structured periplasm. Colonies of *Dichotomicrobium* strains grow slowly; they are rather flat, reddish brown, and have fuzzy edges. Growth in liquid media is pinkish orange; the pigment has been identified as canthaxanthin, a carotenoid (A. Pudleiner, personal communication).

All *Dichotomicrobium* isolates tested by Hirsch and Hoffmann (1989a) require yeast extract for growth, the optimal concentration for five strains ranges from 0.25 to 5.0 g/l. Two of these five strains require Vitamin Solution No. 6 (Van Ert and Staley, 1971). Mass cultures of *Dichotomicrobium* IFAM 954^T have been grown at 43°C with aeration; the yield after 10 d amounted to 7 g dry weight per 7.5 liters. Most strains show growth in media with a 0.2–5.5 fold concentration of artificial seawater (ASW; Lyman and Fleming, 1940), which corresponds to a total salinity of 8–222‰. The optimum ranges from 2.0 to 3.5 × ASW (salinity of 80–142‰). The temperature optimum of nine strains ranges from 37 to 50°C.

Several organic acids are utilized for growth in the presence of 0.025% (w/v) yeast extract; malate is especially favorable, but succinate, 2-oxoglutarate, and acetate also support good growth. Utilization of amino acids, sugars, and sugar alcohols varies with different strains (Table BXII.α.173). Inorganic nitrogen sources do not serve for growth, but yeast extract and some amino acids can be utilized. None of the strains grow with peptone as a nitrogen source. Enzymatic activities are limited. Neither acid nor gas is produced from 0.1% glucose or fructose. Gelatin, starch, casein, and DNA are not hydrolyzed, nitrate is not reduced, and NH₃, H₂S, indole, acetoin, and extracellular phosphatase are not formed. All strains are methyl-red negative, Fe(II) and Mn(II) are not oxidized. The strains are oxidase, catalase, and peroxidase positive.

Chemotaxonomic characteristics of *Dichotomicrobium* strains have been studied by Sittig and Hirsch (1992). Of nine isolates, strains IFAM 951, 954^T, 956, 958, 1185, and 1186 came from the Solar Lake (Sinai) and strains IFAM 1422, 1423, and 1424 from a pond in Brazil. All these isolates have ubiquinone Q-10 and the following phospholipids: phosphatidylglycerol, phosphatidylcholine, bisphosphatidylglycerol, and phosphatidyl-dimethylethanolamine. The three strains from Brazil also contain phosphatidylethanolamine. The fatty acid distribution is also typical (and alike) for the Solar Lake strains, but differs in the Brazilian isolates (Tables BXII.α.174 and BXII.α.175). The fatty acids C_{20:1} 3OH, C_{22:0}, and C_{22:1} ω7 are especially characteristic of the genus *Dichotomicrobium*. A relatively high percentage of C_{19:0} ω7c has been found in strains IFAM 954^T and 1423 (35.2% and 13.5%, respectively); this fatty acid has also been found in other budding, hyphal bacteria, namely *Filomicrobium fusiforme* and *Pedomicrobium* sp. E 1129.

The DNA base compositions of strains IFAM 954^T, 958, and 1185 have been determined to range from 62.7 to 63.6 mol% G + C (T_m; Gebers et al., 1985). The intragenetic heterogeneity (nucleotide distribution) of three *Dichotomicrobium* strains and

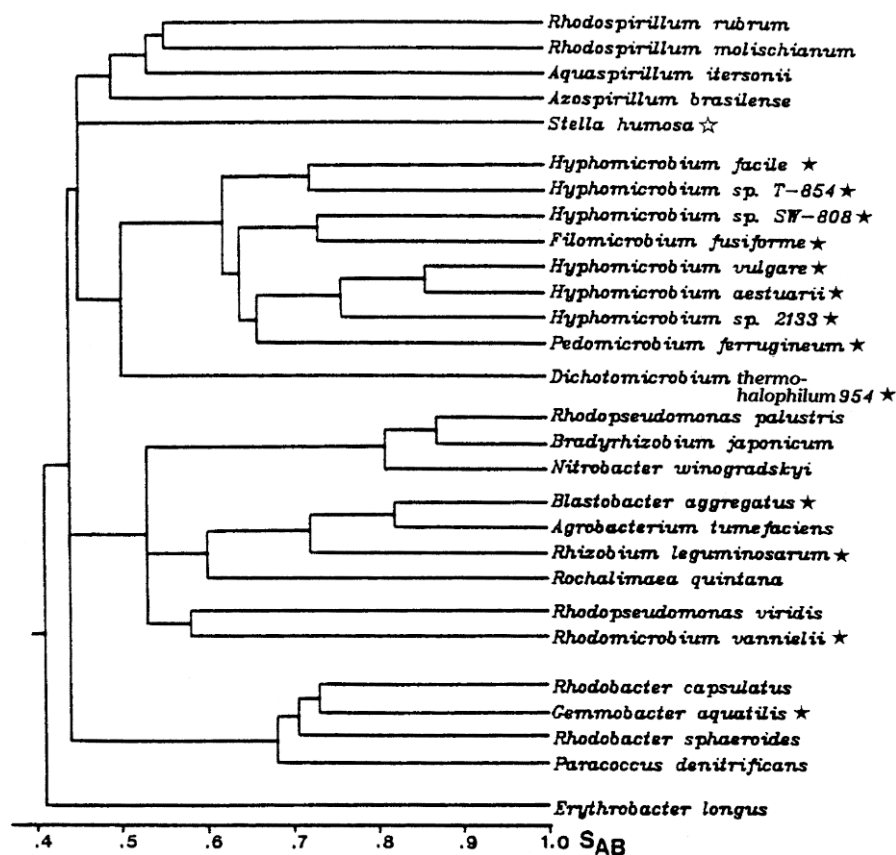


FIGURE BXII.α.205. 16S rRNA dendrogram showing the phylogenetic positions of *Dichotomicrobium thermohalophilum* IFAM 954^T and other budding and/or prosthecae members of the *Alphaproteobacteria*. open star, budding and prosthecae; closed star, prosthecae (Reproduced with permission from E. Stackebrandt et al., Archives of Microbiology 149: 547-556, 1988, ©Springer-Verlag, Berlin.)

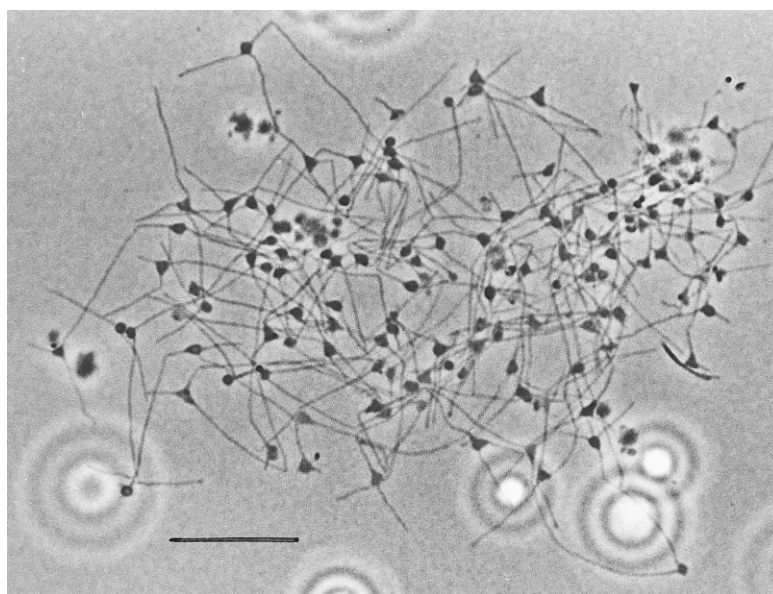


FIGURE BXII.α.206. *Dichotomicrobium thermohalophilum* IFAM 954^T grown in PYGV + 2.5 × ASW at 35°C for 19 d. Phase-contrast light micrograph. Bar = 10 μm.

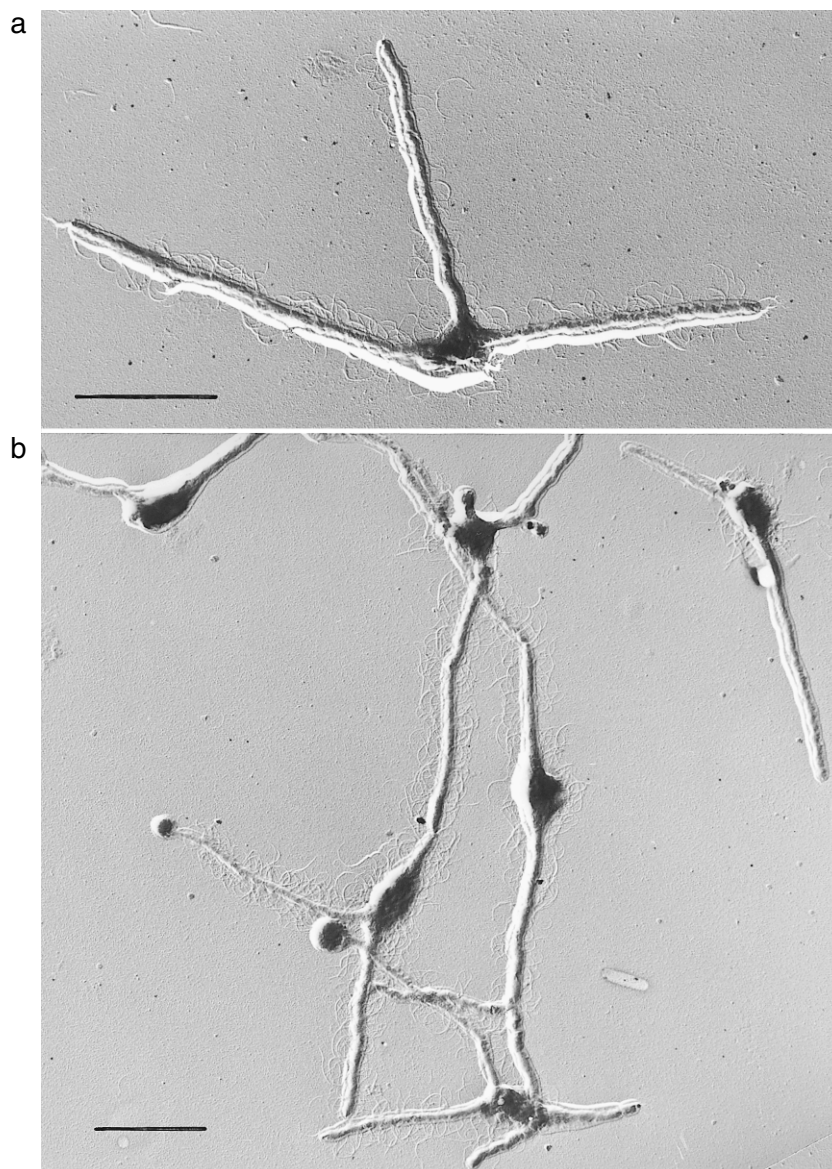


FIGURE BXII.α.207. *Dichotomicrobium thermohalophilum* IFAM 954^T grown 9 d in PYGV + 2.5 × ASW at 43°C. Shadow-cast electron microscopic preparation. (a), young cell with short stubby pili on hyphae; Bar = 2 μm. (b), cells with hyphal branches and terminal buds; Bar = 2 μm (Reproduced with permission from P. Hirsch, Distribution and pure culture studies of morphologically distinct Solar Lake microorganisms. In Nissenbaum (Editor), Hypersaline Brine and Evaporitic Environments, ©Elsevier Science B.V, pp 41–60.)

differences among these and closely related genera (*Pedomicrobium*, “genus T”) have also been studied by plotting DNA base compositions against the widths of the melting transitions. The three *Dichotomicrobium* strains share a high level of similarity with respect to their DNA properties. The genome size of *Dichotomicrobium* strain IFAM 954^T has been determined by DNA renaturation kinetics and found to be 1.73×10^9 bp. When plotted against the mol% G + C ratio, strain 954^T was found to be widely separated from *Hyphomonas*, *Hyphomicrobium*, and *Filomicrobium* species (Kölbel-Boelke et al., 1985).

Antibiotic sensitivity of *Dichotomicrobium* for four antibiotics has been tested by Hirsch and Hoffmann (1989a), with liquid cultures containing 1–100 μg antibiotic per ml. Significant inhibition occurs with 1–10 μg/ml penicillin G or streptomycin sulfate or with 10–100 μg/ml ampicillin or cephalothin.

Bacteria with a *Dichotomicrobium*-like morphology have so far been isolated only from the hypersaline Solar Lake (Sinai) and the Lagoa Vermelha pond in Brazil, which have salinities ranging from 68.7 to 183.3‰ (Table BXII.α.176) and temperatures of 30°C and higher. However, Güde et al., (1985) have observed similar bacterial morphotypes in Lake Constance between 10 and 150 m depth and at a temperature of only 4.5°C. Unfortunately, all attempts to cultivate these forms have failed.

Conditions for Solar Lake dichotomicrobia during sampling time varied considerably within the profile: from 43°C and 144.4‰ salinity near the aerobic surface, through 53°C and 156‰ at 2.5 m depth, to the anaerobic sediment (rich in sulfide) with 42°C and 183.3‰ salinity (Hirsch, 1980; Table BXII.α.176). The Solar Lake dichotomicrobia are evidently well adapted to tolerate this range of conditions.

TABLE BXII.α.173. Some physiological properties of selected *Dichotomicrobium* isolates^{a,b}

Characteristic	IFAM 951	IFAM 954 ^T	IFAM 955	IFAM 958	IFAM 1185	IFAM 1186
<i>C-sources (0.1%, w/v):</i>						
Malate	+	+	+	+	+	+
Glucose	(+)	+	—	nd	nd	nd
<i>C-sources (0.01%, w/v):</i>						
Serine	—	—	+	—	—	—
L-Aspartate	+	+	+	—	+	+
L-Glutamate	+	+	(+)	+	+	—
Peptone	—	—	nd	nd	nd	nd
Yeast extract	+	+	nd	nd	nd	nd
<i>C-sources (0.2%, w/v) (+ 0.25 g/l yeast extract):</i>						
Glucose	+	+	—	—	—	+
Fructose	+	+	—	+	+	+
Mannose	+	+	—	+	—	+
Galactose	+	+	—	+	—	—
Ribose	+	+	—	+	+	—
Arabinose	—	—	—	+	—	—
Mannitol	—	—	+	+	—	—
<i>N-sources (0.1%, w/v) (+ 0.1%, w/v, malate):</i>						
(NH ₄) ₂ SO ₄ , urea	—	—	—	—	—	—
KNO ₂ , KNO ₃	—	—	—	—	—	—
L-Glutamate/L-aspartate	+	+	—	—	+	—
L-Serine	—	(+)	—	—	—	—
Methionine	+	+	—	—	+	+
Arginine, cysteine, leucine	+	+	—	—	+	—
Yeast extract	+	+	+	+	+	+
Peptone	—	—	—	—	—	—

^aSymbols: +, substrate utilized; —, substrate not utilized; (+), growth only weak and slow; nd, not determined.

^bData from Hirsch and Hoffmann (1989a).

TABLE BXII.α.174. Non-hydroxy fatty acids (%) of representative *Dichotomicrobium* strains^a

Fatty acid	<i>D. thermohalophilum</i> IFAM 954 ^T	<i>Dichotomicrobium</i> sp. IFAM 1423
C _{14:1} ω ₁₂	0.5	0.1
C _{14:1} ω ₁₁	0.2	0.1
C _{14:0}	0.1	0.1
C _{16:1} ω ₇	0.2	0.2
C _{16:0}	2.2	2.1
C _{17:0}	0.1	0.1
C _{18:1} ω ₇	39.3	57.8
C _{18:0}	15.2	18.7
C _{18:1} ω ₆ 11CH ₃	1.4	1.4
C _{19:0} cyclo ω _{7c}	35.2	13.5
C _{20:1} ω ₇	4.7	4.9
C _{20:0}	0.2	0.3
C _{21:0}	—	0.2
C _{22:1} ω ₇	0.1	0.1
C _{22:0}	0.1	—

^aData from Sittig and Hirsch (1992).

TABLE BXII.α.175. Hydroxy fatty acids (%) of representative *Dichotomicrobium* strains^a

Hydroxy fatty acid	<i>Dichotomicrobium</i> IFAM 954 ^T	<i>Dichotomicrobium</i> IFAM 1423
C _{14:0} 3OH	10.8	3.1
C _{16:0} 3OH	2.9	1.8
C _{18:1} 3OH	3.5	5.7
C _{18:0} 3OH	72.1	73
C _{20:1} 3OH	9.9	15.2
C _{20:0} 3OH	0.7	1.2

^aData from Sittig and Hirsch (1992).

Three strains have been isolated from enrichments kept under N₂/CO₂ (95:5), but when tested, these isolates could not grow anaerobically. Since viable dichotomicrobia have been obtained from the anaerobic enrichments and from the anaerobic sediment, a lack of oxygen must be tolerated by these bacteria at least for some time. Survival and limited growth in the an-

aerobic enrichments could have been made possible by the presence of oxygen-producing cyanobacteria and dim light.

ENRICHMENT AND ISOLATION PROCEDURES

Two methods have successfully yielded *Dichotomicrobium* cultures. (1) Clean, sterile glass slides are exposed to the Solar Lake for

TABLE BXII.α.176. Origin and enrichment conditions of 13 strains of *Dichotomicrobium* sp.^a

Characteristic	IFAM 951, 952	IFAM 953, 954 ^T	IFAM 955, 956	IFAM 957, 958	IFAM 1185	IFAM 1186	IFAM 1422	IFAM 1423	IFAM 1424
<i>Sample origin:</i>									
Solar Lake (Sinai)	+	+	+	+	+	+			
Lagoa Vermelha (Brazil)							+	+	+
Depth (m)	2.5	3.5	1.5	1.5	4.3	2	0.2	0.2	0.2
Salinity (‰)	156.2	169.1	144.4	144.4	183.3	151.2	86.5	68.7	86.5
<i>Enrichment conditions:</i>									
Temperature (°C)	43	43	43	43	43	43	30	30	30
Atmosphere	O ₂	N ₂	O ₂	O ₂	N ₂	N ₂	O ₂	O ₂	O ₂
<i>Additions:</i>									
Malate							+	+	+
Peptone			+						
Yeast extract and Vitamin solution No. 6	+								
Medium pH	7.2	7.2	7.2	7.2	7.2	7.2	8.5	7.2	8.5
<i>Optimal conditions for growth:</i>									
Salinity (‰)	121	142	111	111	121	142	80	80	80
Temperature (°C)	49	50	50	50	44	48	45	37	38
pH	nd ^b	8.3	nd	nd	nd	nd	8.5	7.1	8.3

^aData from Hirsch and Hoffmann (1989a).^bnd, not determined.

8–10 d to allow dichotomicrobia to attach. The slides are then transferred to a sample of Solar Lake water. In the laboratory, samples and slides are incubated either directly or with the addition of 0.025% (w/v) of Bacto yeast extract (sterilized in the same water). Alternatively, Bacto peptone is added to other samples with exposed slides. The samples (enrichments) are then incubated up to four weeks at 30–45°C (Hirsch, 1980; Hirsch and Hoffmann, 1989a). (2) The other successful procedure consists of directly streaking water samples containing dichotomicrobia onto agar plates with medium 398, of the following composition (g/l): Bacto yeast extract, 1; DL-malate, 1; Hutner's basal salts (Cohen-Bazire et al., 1957), 20 ml; 3× ASW, 980 ml; final pH 7.2. Some strains grow better on medium 399 with the same ingredients, but only 2.5× concentrated ASW and a final pH of 8.5. Identification and isolation of dichotomicrobia on the same media is facilitated by the reddish-brown color and fuzzy edges of the individual colonies.

MAINTENANCE PROCEDURES

Pure cultures can be stored in liquid medium at 20°C for at least one year, but storage at 4°C is much less effective. Lyophilization in growth medium (398, 399) (see Enrichment and Isolation Procedures above) is better than in skim milk. Slants kept at 20°C must be subcultured every 4–6 months, but liquid cultures survive better, as long as medium evaporation is controlled.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Mass cultures for the isolation of DNA contain (g/l): Bacto peptone, 1; Bacto yeast extract, 1; glucose, 1; vitamin solution no. 6, 10 ml; Hutner's basal salts, 20 ml; ASW (2.5–3×), 970 ml; final pH 7.5 (Gebers et al., 1985). Cells are harvested at 16,000× g for 20 min, washed twice with 0.85% (w/v) NaCl, and the pellet stored at –20°C for further use.

Lysis of dichotomicrobia is achieved by suspending 1 g (wet weight) of cells in 20 ml of 1 M NaCl/0.1 M EDTA containing 1 g proteinase K and 1% (w/v) *N*-acetyl-*N,N,N*-trimethylammonium bromide (E. Merck), as well as 50 g of glass beads (Gebers

et al., 1985). This mixture is treated for 5 sec in the cell homogenizer, followed by the addition of 1 volume chloroform–isoamyl alcohol and shaking at 100 rpm for 15 min. The resulting emulsion is centrifuged at 1350× g for 20 min; the upper aqueous phase is then removed and mixed with 1 volume double distilled water. Then, 0.6 volume of isopropanol is added, drop by drop, while the solution is stirred with a glass rod. Centrifugation at 1350× g for 10 min yields a nucleic acid pellet, which can be further purified as described by Marmur (1961).

DIFFERENTIATION OF THE GENUS *DICHOTOMICROBIUM* FROM OTHER GENERA

Differentiation of the genus *Dichotomicrobium* from other hyphal and budding bacteria is shown on Table BXII.α.177.

TAXONOMIC COMMENTS

Ten *Dichotomicrobium* strains came from the Solar Lake, with IFAM 954^T as a representative isolate. Three other strains from the shallower Lagoa Vermelha (Brazil) are physiologically slightly different; a representative strain is IFAM 1423. These isolates have lower salinity and temperature optima (Table BXII.α.176), and they have different percentages of non-hydroxy (Table BXII.α.174) and hydroxy fatty acids (Table BXII.α.175). Additionally, the average cell size of these Brazilian strains was lower. For these reasons, only the ten nearly identical strains IFAM 951–1186 are described as *D. thermohalophilum* (Hirsch and Hoffmann, 1989a) and the Lagoa Vermelha isolates have been recommended for further studies as possibly different species.

ACKNOWLEDGMENTS

Skillful technical assistance rendered by B. Hoffmann and M. Beese is gratefully acknowledged. A. Höhn (Geesthacht) provided the Lagoa Vermelha water samples and physical data on the habitat. H. Güde (Langenargen) and M. Simon (Konstanz) contributed information on *Dichotomicrobium*-like morphotypes in Lake Constance. The Solar Lake studies were initiated by the late Prof. M. Shilo (Jerusalem) and supported by the Deutsche Forschungsgemeinschaft.

TABLE BXII.α.177. Differentiation of *Dichotomicrobium* from other related genera of budding and hyphal bacteria

Characteristics	<i>Dichotomicrobium</i> ^a	<i>Pedomicrobium</i> ^b	<i>Filomicrobium</i> ^c	<i>Hirschia</i> ^d	<i>Hyphomicrobium</i> ^e	<i>Hyphomonas</i> ^f	<i>Rhodomicrobium</i> ^g
<i>Cell shape:</i>							
Tetrahedral	+	V ^h	—	—	—	—	V
Ovoid, pear-, bean-shaped	V	+	—	+	+	+	+
Fusiform	—	—	+	—	—	—	—
Intracytoplasmic membranes	—	V	—	—	V	—	+
PHBA in mother cells and/or hyphae	+ / +	- / -	- / -	- / -	+ / -	—	+ / -
Bud perpendicular on hyphal tip	—	+	—	—	—	—	—
Daughter cells motile	—	+	—	+	+	+	+
Colony pigmentation ⁱ	Rb	Db	Lr	Y	Bb	C	R
Salinity range for growth (‰ ASW)	80–142	1 to <10	4.3–34.5	4.3–86.3	0–25 ^j	5–150 ^j	nd ^k
Anaerobic growth	—	—	—	—	d	—	V
Fe ²⁺ or Mn ²⁺ oxidized	—	+	—	—	V	—	—
Main respiratory quinone component	Q-10	Q-10	Q-9	Q-10	Q-9	Q-11/Q-10	Q-10
Mol% G + C (<i>T_m</i>)	62–64	62–67	62	46–47	59–65	57–62	62–64

^aData from Hirsch and Hoffmann (1989a).^bData from Gebers (1981).^cData from Schlesner (1987).^dData from Schlesner et al., (1990).^eData from Hirsch (1989).^fData from Weiner et al., (1985).^gData from Duchow and Douglas (1949).^hV denotes occasional mesosomal structures.ⁱRb, reddish brown; Db, dark brown; Lr, light red; Y, yellow; Bb, brownish to beige; C, colorless; R, red.^jNaCl salinity.^knd, not determined.*List of species of the genus Dichotomicrobium*

1. ***Dichotomicrobium thermohalophilum*** Hirsch and Hoffmann 1989b, 495^{VP} (Effective publication: Hirsch and Hoffmann 1989a, 300.)
ther.mo.ha.lo'phi.lum. Gr. adj. *thermus* hot; Gr. n. *halus* salt; Gr. adj. *philus* loving; M.L. neut. adj. *thermohalophilum* heat and salt loving.

Morphological description is the same as for the genus. Temperature optimum for growth 44–50°C, temperature minimum 20–30°C, temperature maximum 61–65°C. Temperature survival range 13–65°C. Optimal pH 8.0–8.5, pH range for growth 5.8–9.5. Optimal salinity for growth 80–

142‰ ASW, minimal salinity 8 to >40‰, maximal salinity 182 to >222‰. Optimal concentration of yeast extract 0.25–5.0 g/l (w/v). Other physiological and biochemical characteristics as for the genus. Habitat: hypersaline, meromictic, and heliothermal Solar Lake (Sinai), throughout the whole profile (0–4.5 m).

The mol% G + C of the DNA is: 62–64 (*T_m*).

Type strain: IFAM 954, ATCC 49408, DSM 5002.

Additional Remarks: Reference strains include IFAM 951, 952 (DSM 5001), 953, 955, 956, 957, 958, 1185, and 1186 (DSM 5006).

Genus X. *Filomicrobium* Schlesner 1988, 220^{VP} (Effective publication: Schlesner 1987, 65)

HEINZ SCHLESNER

Fi.lo.mi.cro'bi.um. L. masc. n. *filum* thread; Gr. adj. *micros* small; Gr. n. *bios* life; M.L. neut. n. *Filomicrobium* threadlike microbe.

Fusiform cells, 0.5–0.7 × 1.5–4.0 μm, with two or three polar prosthecae, which are about 0.2 μm in diameter and have a length of up to 40 μm or more. Buds are formed at the tips of the prosthecae (Fig. BXII.α.208). **Nonmotile. Gram negative. Aerobic and chemoorganotrophic.** Utilize some organic acids. **C₁-compounds are not used as carbon sources.** The ubiquinone system is a **Q-9** system. Hydroxy fatty acids are of the **3-OH** type.

The mol% G + C of the DNA is: 62.

Type species: ***Filomicrobium fusiforme*** Schlesner 1988, 220 (Effective publication: Schlesner 1987, 65.)

FURTHER DESCRIPTIVE INFORMATION

Growth on solid media is poor. After 3 weeks of incubation at 25°C, microcolonies with diameters of about 0.1 mm can be ob-

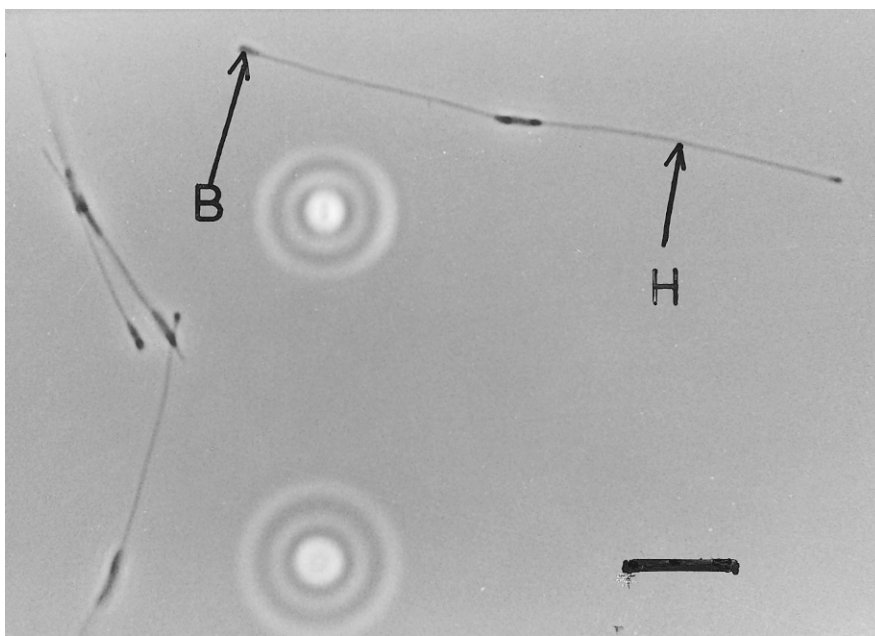


FIGURE BXII.α.208. *Filomicrobium fusiforme* IFAM 1315^T. Cells with polarly inserted hyphae (H) at the tips of which buds (B) are being formed. Bar = 10 μm.

served. They are light red and have a rough surface. The red pigment is composed of canthaxanthin and echinone and can be extracted with ethanol and methanol but not with chloroform or petroleum ether. The genome size is 2.03×10^9 Da (Kölbel-Boelke et al., 1985).

Analysis of the 16S rDNA indicates that the genus *Filomicrobium* is a member of the *Alphaproteobacteria* and is related to two clusters of species of the genus *Hyphomicrobium* (Rainey et al., 1998).

ENRICHMENT AND ISOLATION PROCEDURES

The type strain was isolated from a sample taken from the Kiel Bight (part of the Baltic Sea, Germany) at a depth of 2 m. For enrichment, the sample was incubated at 20–23°C in daylight for 12 months and then streaked on agar plates of medium 17 (M17): sodium acetate, 1.0 g; KNO₃, 1.0 g; Hunter's basal salts (Cohen-Bazire et al., 1957), 20 ml; vitamin solution (Staley, 1968), 10 ml; artificial sea water (Lyman and Fleming, 1940), 500 ml; distilled water to 1000 ml (Schlesner, 1987).

MAINTENANCE PROCEDURES

Storage on solid media is not recommended. The cells are easily revived from lyophilized cultures.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Cells of *Filomicrobium* are easily recognized by microscopy.

DIFFERENTIATION OF THE GENUS *FILOMICROBIUM* FROM OTHER GENERA

Table BXII.α.55 in the entry describing the genus *Hirschia* lists the major features that differentiate *Filomicrobium* from other genera of budding, prosthecate bacteria.

FURTHER READING

Schlesner, H. 1987. *Filomicrobium fusiforme* gen. nov., sp. nov., a slender budding, hyphal bacterium from brackish water. Syst. Appl. Microbiol. 10: 63–67.

List of species of the genus *Filomicrobium*

1. ***Filomicrobium fusiforme*** Schlesner 1988, 220^{VP} (Effective publication: Schlesner 1987, 65.)
fu.si.for' me. L. n. *fusus* spindle; L. n. *forma* shape, form; M.L. adj. *fusiformis* spindle-shaped.

Poor growth on solid media. Microcolonies 0.1–0.2 mm in diameter, with a rough surface, occur after 3 weeks incubation at 25°C. Growth is optimal between 20°C and 28°C; maximum growth temperature is 33°C. Vitamin B₁₂ is required. Require eighth to full strength seawater for growth; optimal growth is with half-concentrated seawater. Carbon

sources utilized for growth are acetate, propionate, fumarate, malate, succinate, and glutamic acid. Glucose is not fermented; nitrate is not reduced. Alginate, cellulose, chitin, and starch are not hydrolyzed. Ammonia, formamide, nitrate, and urea can serve as nitrogen sources. See Table BXII.α.178 for additional characteristics. Red pigment in type strain. Habitat is brackish water.

The mol% G + C of the DNA is: 62 (T_m).

Type strain: SH 128, ATCC 35158, DSM 5304, IFAM 1315.

GenBank accession number (16S rRNA): Y14313.

TABLE BXII.α.178. characteristics of *Filomicrobium fusiforme*

Characteristic	Reaction/Result
<i>Carbon source utilization:</i>	
D-Arabinose	—
L-Arabinose	—
Cellobiose	—
Fructose	—
Galactose	—
Glucose	—
Lactose	—
Lyxose	—
Maltose	—
Mannose	—
Melibiose	—
Raffinose	—
Rhamnose	—
Ribose	—
Sorbose	—
Sucrose	—
Trehalose	—
Xylose	—
Methanol	—
Ethanol	—
Glycerol	—
Erythritol	—
Adonitol	—
Arabitol	—
Dulcitol	—
Inositol	—
Mannitol	—
Sorbitol	—
Acetate	+
Adipate	—
Benzoate	—
Butyrate	—
Caproate	—
Citrate	—
Formate	—
Fumarate	+
Glutarate	—
Lactate	—
Malate	+
Oxalate	—
2-Oxoglutarate	—
Phthalate	—
Propionate	+
Pyruvate	—
Salicylate	—
Succinate	+
Tartrate	—
Valerate	—
L-Alanine	—
L-Arginine	—
L-Asparagine	—
L-Aspartic acid	—
Glutamic acid	+
L-Glutamine	—
Glycine	—
L-Histidine	—

(continued)

TABLE BXII.α.178. (cont.)

Characteristic	Reaction/Result
L-Isoleucine	—
L-Leucine	—
L-Lysine	—
L-Methionine	—
D,L-Norleucine	—
L-Ornithine	—
L-Phenylalanine	—
L-Proline	—
L-Serine	—
L-Threonine	—
L-Tryptophan	—
L-Tyrosine	—
L-Valine	—
N-Acetylglucosamine	—
Amygdalin	—
Dextrin	—
Gluconate	—
Glucosamine	—
Glucuronate	—
Glycogen	—
Inulin	—
Pectin	—
Salicin	—
<i>Utilization of nitrogen sources:</i>	
N-Acetylglucosamine	—
Ammonia	+
Formamide	+
Nicotinic acid	—
Nitrate	+
Urea	+
<i>Hydrolysis of:</i>	
Alginate	—
Cellulose	—
Chitin	—
Starch	—
Fermentation of glucose	—
Hemolysis of horse blood	—
Ammonia produced from peptone	—
H ₂ S produced from thiosulfide	—
Catalase	+
Cytochrome oxidase	+
Urease	+
<i>Sensitive to 1 µg/ml of:</i>	
Chloramphenicol	+
Penicillin G	+
Streptomycin	+
Tetracycline	+
<i>Fatty acids:</i>	
C _{18:1 ω7}	+
C _{18:2 ω7, 13}	+
C _{19:0 cyclo ω7c}	+
<i>Phospholipids:</i>	
Phosphatidylglycerol (PG)	+
Phosphatidylethanolamine (PE)	—
Phosphatidylmonomethylethanolamine (MPE)	—
Phosphatidyltrimethylethanolamine (DPE)	+
Phosphatidylcholine (PC)	—
Bisphosphatidylglycerol (BPG)	+

Genus XI. **Gemmiger** Gossling and Moore 1975, 206^{AL}

JENNIFER GOSSLING

Gem'mi.ger. L. n. *gemma* a bud; L. v. *gero* to bear; M.L. masc. n. *Gemmiger* bud bearer.

Ovoid to hourglass-shaped bacteria, 0.9–2.5 × 1.0 µm, that apparently divide at a constriction, giving the **appearance of budding**. Rapidly growing organisms may form chains. Gram variable to Gram negative. Do not form spores. **Nonmotile** and with no external structures.

Obligately **anaerobic chemoorganotrophs using carbohydrate** as the only or major energy source. Growth on glucose or other sugars produces **butyrate**, usually lactate and formate, and sometimes small amounts of other compounds. Catalase-negative.

The mol% G + C of the DNA is: 59.

Type species: Gemmiger formicilis Gossling and Moore 1975, 206.

FURTHER DESCRIPTIVE INFORMATION

The cells most commonly have a bowling-pin shape and are 0.9–2.5 μm long; the diameter of the larger end is 0.5–1.0 μm , that of the smaller end is 0.2–0.8 μm , and that of the constriction is 0.2–0.5 μm (Figs. BXII.α.209 and BXII.α.210). The constriction may not be resolved under the light microscope, and the organism is seen as a pair of cocci, usually with one smaller than the other, resembling a budding yeast. Where the constriction is relatively long, secondary constrictions may be seen by electron microscopy. The bacteria are often seen in pairs, especially in logarithmic phase cultures; the two bacteria are attached to each other by the smaller ends (Figs. BXII.α.210 and BXII.α.211). Long chains are also formed, including pairs with the smaller ends together. At low concentrations of penicillin, sufficient to inhibit division but not growth, long filaments are formed (Salanitro et al., 1976).

There is a multilayered cell wall (Fig. BXII.α.211) (Gossling and Moore, 1975; Salanitro et al., 1976). Intracellular granules of a glycogen-like substance may be found (Salanitro et al., 1976).

Colonies are 1–2 mm in diameter after 48 h of growth. They are usually circular, entire, smooth, and low convex. They may be clear, translucent or opaque cream or white, depending on the medium. Broth cultures may become turbid initially, but the growth usually settles to form a ropy sediment.

Strict anaerobes, they have been cultured by using the roll tube, glove box, and steel wool (Mitsuoka, 1980) techniques but

cannot be cultured on agar medium that has been exposed to air and is incubated in a conventional anaerobic jar (Gossling and Moore, 1975; Croucher and Barnes, 1983). Carbon dioxide is normally added to the gas phase for incubation and appears necessary for good growth.

Carbohydrates or related compounds are required for growth. Most strains use glucose; fresh isolates—from humans—that do not use glucose will use maltose (Holdeman et al., 1976; Mitsuoka, 1980). For strains from chickens, NH_4^+ has been shown to be the preferred nitrogen source (Salanitro et al., 1976; Croucher and Barnes, 1983). Various unidentified factors present in ruminal, fecal, liver or yeast extracts are necessary for growth. Growth of strains from chickens is stimulated by thiamine, riboflavin, pantothenate, and straight chain volatile fatty acids (Salanitro et al., 1976). Growth of some strains from humans is stimulated by Tween 80 (0.01–0.10%), but this is inhibitory to other strains (Gossling and Moore, 1975; Salanitro et al., 1976).

Glucose is fermented with the production of butyrate and usually formate and lactate; other products may include acetate, pyruvate, succinate, and malonate. All strains use and ferment a range of the following substances: amygdalin, arabinose, arabinoxylan, cellobiose, dextrin, esculin, fructose, galactose, glucose, glycogen, inulin, lactose, maltose, mannose, melibiose, raffinose, salicin, starch, sucrose, trehalose, and xylose. Lactate and nitrogenous compounds are not fermented.

A chicken isolate that has been tested for antibiotic susceptibility in broth was resistant to tetracycline (10 $\mu\text{g}/\text{ml}$), doxycycline (10 $\mu\text{g}/\text{ml}$), vancomycin (2.5 $\mu\text{g}/\text{ml}$), erythromycin

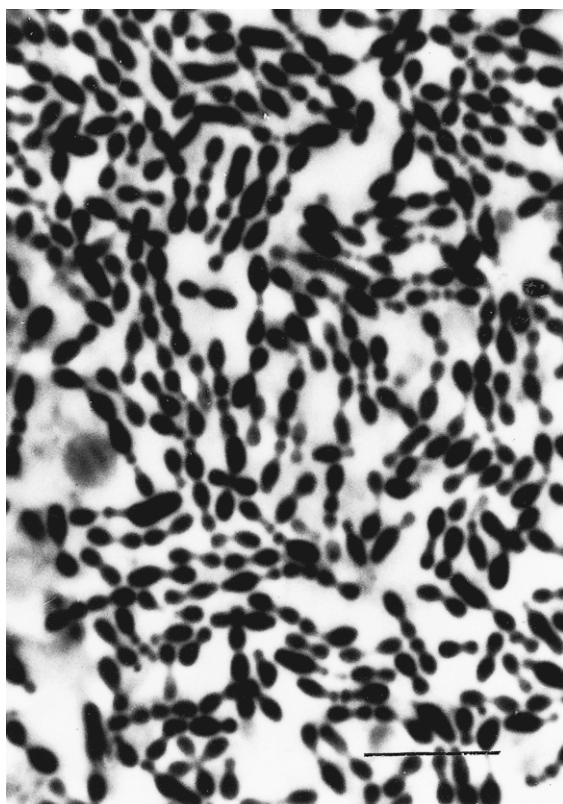


FIGURE BXII.α.209. *G. formicilis* strain SC3/5. Phase-contrast photomicrograph of broth culture. Bar = 5 μm . (Reproduced with permission from S.C. Croucher and E.M. Barnes, *Revue de l'Institut Pasteur de Lyon* 14: 95–102, 1981, ©Institut Pasteur de Lyon.)

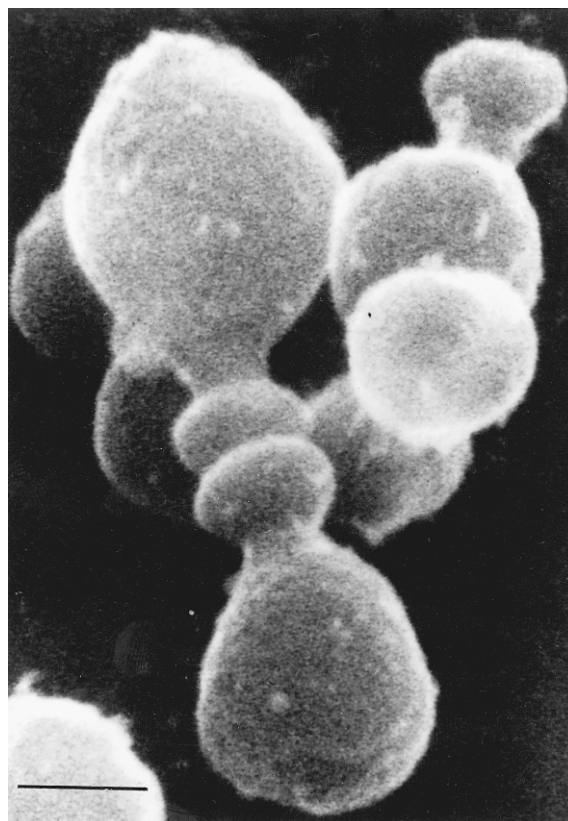


FIGURE BXII.α.210. *G. formicilis* strain L61. Scanning electron micrograph. Bar = 0.5 μm . (Reproduced with permission from J. Gossling and W.E.C. Moore, *International Journal of Systematic Bacteriology* 25: 202–207, 1975, ©International Union of Microbiological Societies.)

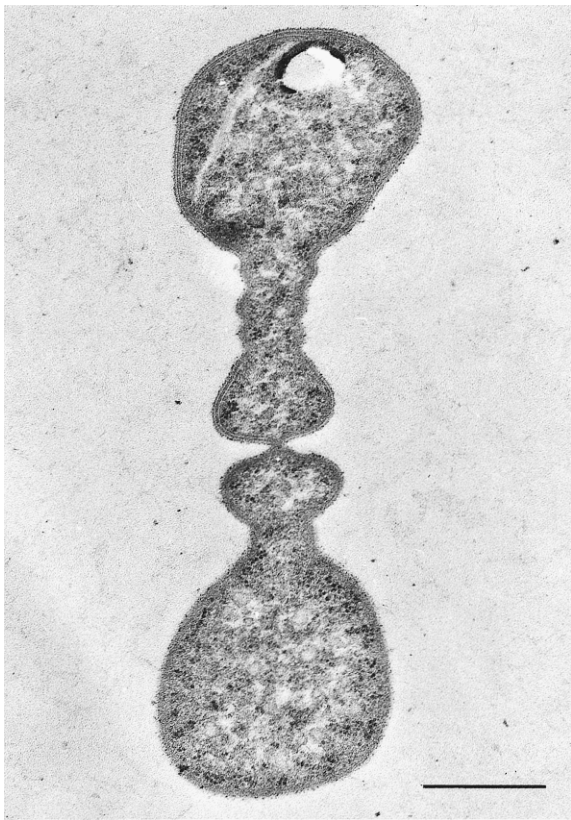


FIGURE BXII.α.211. *G. formicilis* strain L61. Transmission electron micrograph of a thin section. Bar = 0.5 μm. (Reproduced with permission from J. Gossling and W.E.C. Moore, International Journal of Systematic Bacteriology 25: 202–207, 1975, ©International Union of Microbiological Societies.)

(5 μg/ml), kanamycin (100 μg/ml), neomycin (100 μg/ml), and nalidixic acid (100 μg/ml); it was inhibited by tetracycline (100 μg/ml), doxycycline (100 μg/ml), vancomycin (5 μg/ml), erythromycin (10 μg/ml), and clindamycin (1 μg/ml) (Croucher and Barnes, 1983).

Gemmiger isolates have been obtained from chickens and humans. In chickens up to 6 weeks of age, these bacteria live in the lumen of the large intestine and the ceca, forming a significant proportion—often >10%—of the flora (Salanitro et al., 1976; Croucher and Barnes, 1983). In adult humans they comprise about 2% of the fecal bacteria, giving counts of about 10^{10} /g dry wt (Moore and Holdeman, 1974; Gossling and Moore, 1975; Holdeman et al., 1976). They presumably live on residual carbohydrates that are not digested or absorbed in the small intestine.

List of species of the genus *Gemmiger*

1. ***Gemmiger formicilis*** Gossling and Moore 1975, 206^{AL}
for.mi'ci.lis. M.L. adj. *formicilis* pertaining to formic acid.

Strictly anaerobic, mesophilic bacteria conforming to the generic description. Require glucose, fructose, maltose, or other carbohydrate-like substances (see generic description) for growth. Produce butyrate and usually formate and lactate and no gas from glucose and other sugars. The predominant product varies with the strain and the sugar used. The terminal pH in weakly buffered media is 4.8–6.0. Er-

ENRICHMENT AND ISOLATION PROCEDURES

Via the roll tube technique, *Gemmiger* strains are isolated on media enriched with ruminal fluid, fecal extract, or liver extract and containing carbohydrates (glucose, maltose and/or cellobiose, soluble starch). No successful selection or enrichment techniques have been reported. Samples are diluted under strictly anaerobic conditions and streaked out on agar media for isolated colonies. After incubation for 3–7 days, large samples of colonies are picked for identification. *Gemmiger* can be distinguished microscopically by using phase contrast (Fig. BXII.α.209) or Gram stain (Mitsuoka, 1980).

MAINTENANCE PROCEDURES

Gemmiger strains are maintained by lyophilization, via methods suitable for strict anaerobes.

DIFFERENTIATION OF THE GENUS *GEMMIGER* FROM OTHER GENERA

The key feature that distinguishes *Gemmiger* from aberrant minicell-producing bacilli, budding cocci, or streptococci is the appearance of two small forms between two large ones (Fig. BXII.α.209). Metabolically, *Gemmiger* can be distinguished from most other intestinal anaerobes by their requirement for carbohydrates together with the production of butyrate and no gas.

TAXONOMIC COMMENTS

Gemmiger does not fit into any described higher taxon. Metabolically, these bacteria have been compared with other anaerobic intestinal bacilli, such as *Eubacterium* and the *Bacteroidaceae* (Moore and Holdeman, 1974; Salanitro et al., 1976), but *Gemmiger* has a higher mol% G + C of the DNA than do any of these. The mode of division may be similar to that of the facultative, prosthecate, freshwater bacterium *Ancalomicrobium* (Staley, 1968), but this bacterium has a higher mol% G + C of the DNA than does *Gemmiger*. Other bacteria that may have a similar mode of division are even more different metabolically (Whittenbury and Nicoll, 1971; Hirsch, 1974a).

Gemmiger have been grouped with the anaerobic cocci (Moore and Holdeman, 1974; Mitsuoka, 1980), but the formation of filaments in sublethal concentrations of penicillin (Salanitro et al., 1976) does not indicate a typical coccal morphology.

FURTHER READING

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- Gossling, J. and W.E.C. Moore. 1975. *Gemmiger formicilis*, n. gen., n. sp., an anaerobic budding bacterium from intestines. Int. J. Syst. Bacteriol. 25: 202–207.
- Salanitro, J.P., P.A. Muirhead and J.R. Goodman. 1976. Morphological and physiological characteristics of *Gemmiger formicilis* isolated from chicken ceca. Appl. Environ. Microbiol. 32: 623–632.

ythritol, inositol, mannitol, melezitose, rhamnose, and ribose are not fermented. Indole, lecithinase, lipase, acetyl-methylcarbinol, hydrogen sulfide, catalase, and urease are not produced. Nitrate is not reduced. Hippurate is not hydrolyzed. Casein and meat are not digested; there may be a weak action on gelatin (Gossling and Moore, 1975).

Study of chicken isolates indicates that most of them belong to one of two groups on the basis of fermentation products and the carbon sources used (Table BXII.α.179)

(Salanitro et al., 1976; Croucher and Barnes, 1983). Human isolates may be divided on the same characters, but the groups obtained do not correspond with the chicken groups.

All chicken isolates use raffinose and salicin; most human strains do not. However, the type strain is a human strain that uses salicin (Gossling and Moore, 1975).

It has been suggested that the human strains, which grow poorly in culture, do not use glucose on initial isolation, and do not produce formate, belong to a separate species (Holdeman et al., 1976); however, strains that normally produce formate may not do so when not growing well (Gossling and Moore, 1975).

The mol% G + C of the DNA is: 59 (T_m).

Type strain: ATCC 27749.

Genus XII. *Labrys* Vasilyeva and Semenov 1985, 375^{VP} (Effective publication: Vasilyeva and Semenov 1984, 92)

LEANA V. VASILYEVA

Lab'rys. Gr. n. *Labrys* double-headed ax, an organism resembling a Minoan ax, by the shape of the cell.

Unicellular flat bacterium; cells possess **triangular radial symmetry**. Dimensions are 1.1–1.3 × 1.3–1.5 µm. **Two to three tapering, short prosthecae** (<0.6 µm) protrude from two corners of the triangle; the third remains free and is associated with multiplication.

Cells divide by budding. Buds are produced directly from the mother cell at the tip of the triangle that lacks prosthecae. In this stage, the cell resembles a double-headed ax or *labrys*.

Gram negative, nonmotile, and do not possess fimbriae.

Obligately aerobic, nonfermentative, **chemoorganotrophic**. Utilize carbohydrates and some organic acids as sole carbon and energy sources. The type strain requires B vitamins for growth. Oxidase and catalase positive. Found in freshwater lakes.

The mol% G + C of the DNA is: 67.9.

Type species: ***Labrys monachus*** Vasilyeva and Semenov 1985, 375 (Effective publication: Vasilyeva and Semenov 1984, 92.)

ENRICHMENT AND ISOLATION PROCEDURES

Strain was isolated from the silt samples from Lake Mustjarv (Estonia). The medium is horse manure extract, obtained by heating dry manure 1% (w/v) in distilled water. The sediment is left to settle, and a liquid medium is prepared from the supernatant; 2% agar is added for solidification and colony isolation. Incubation is at 28°C for 10–14 d.

Minute colonies are isolated and examined under a phase-contrast microscope for the presence of bacteria with unusual morphology. Repeated subculturing of individual colonies produces a pure culture.

List of species of the genus Labrys

1. ***Labrys monachus*** Vasilyeva and Semenov 1985, 375^{VP} (Effective publication: Vasilyeva and Semenov 1984, 92.) *mon.ach'us*. M.L., from ancient Gr. adj. *monachos* the only, unique, single.

The description is as for the genus.

Unicellular, flat, triangular, budding prosthecobacterium. Dimensions 1.1–1.5 µm. Prosthecae are short, <0.6 µm, tapering and protruding from two corners of triangle (Fig. BXII.α.212). Cells nonmotile. Gas vacuoles are not produced.

TABLE BXII.α.179. Differential characteristics of the groups of *Gemmiger formicilis* from poultry^a

Characteristic	Group 1	Group 2
<i>Major fermentation product from glucose:</i>		
Butyrate	—	+
Lactate	+	—
<i>Growth on:</i>		
Cellobiose	+	—
Mannose	+	—
Sucrose	+	—
Trehalose	+	—

^aSymbols: —, 90% or more of strains are negative; and +, 90% or more of strains are positive.

MAINTENANCE PROCEDURES

Potato agar provides a favorable growth medium. Growth occurs on MMB agar (see *Ancalomicrobium*) with glucose, but only if supplemented by up to 0.25% yeast extract (Vasilyeva and Semenov, 1984). The type strain can be maintained on slants in the refrigerator for at least 3 months. Lyophilization can be used for long-term preservation.

DIFFERENTIATION OF THE GENUS *LABRYS* FROM OTHER GENERA

Labrys combines morphological features typical of budding bacteria with prosthecae, such as *Prosthecomicrobium* and *Ancalomicrobium*, and of flat bacteria with radial symmetry, such as *Angulomicrobium* and *Stella* (Vasilyeva, 1980).

Physiological differences among these organisms are minimal.

Labrys, unlike *Angulomicrobium*, possesses prosthecae, which makes differentiation easy, in spite of the resemblance in cell shape and budding. Unlike *Ancalomicrobium*, *Labrys* has flat cells.

TAXONOMIC COMMENTS

Labrys combines features of a number of genera, and inclusion of these organisms into other genera would necessarily change their descriptions. Only a single strain has so far been isolated. DNA–DNA hybridization studies have revealed no homology with *Stella* (Lysenko et al., 1984). Further work is needed to reveal a phylogenetic interrelation among budding prosthecobacteria.

Gram negative. On the outer cell surface is an irregular, visible capsular microlayer (Fig. BXII.α.213). No laminated membranous structures in the cells.

Division is by budding on the corner that is free of prosthecae. The daughter cell separates when it approaches mother-cell size and shape (Fig. BXII.α.214).

Utilizes the following carbohydrates as sole sources of carbon and energy: D-erythrose, D-ribose, L-arabinose, D-xylose, D-lyxose, D-glucose, L-sorbose, L-rhamnose, D-fructose.

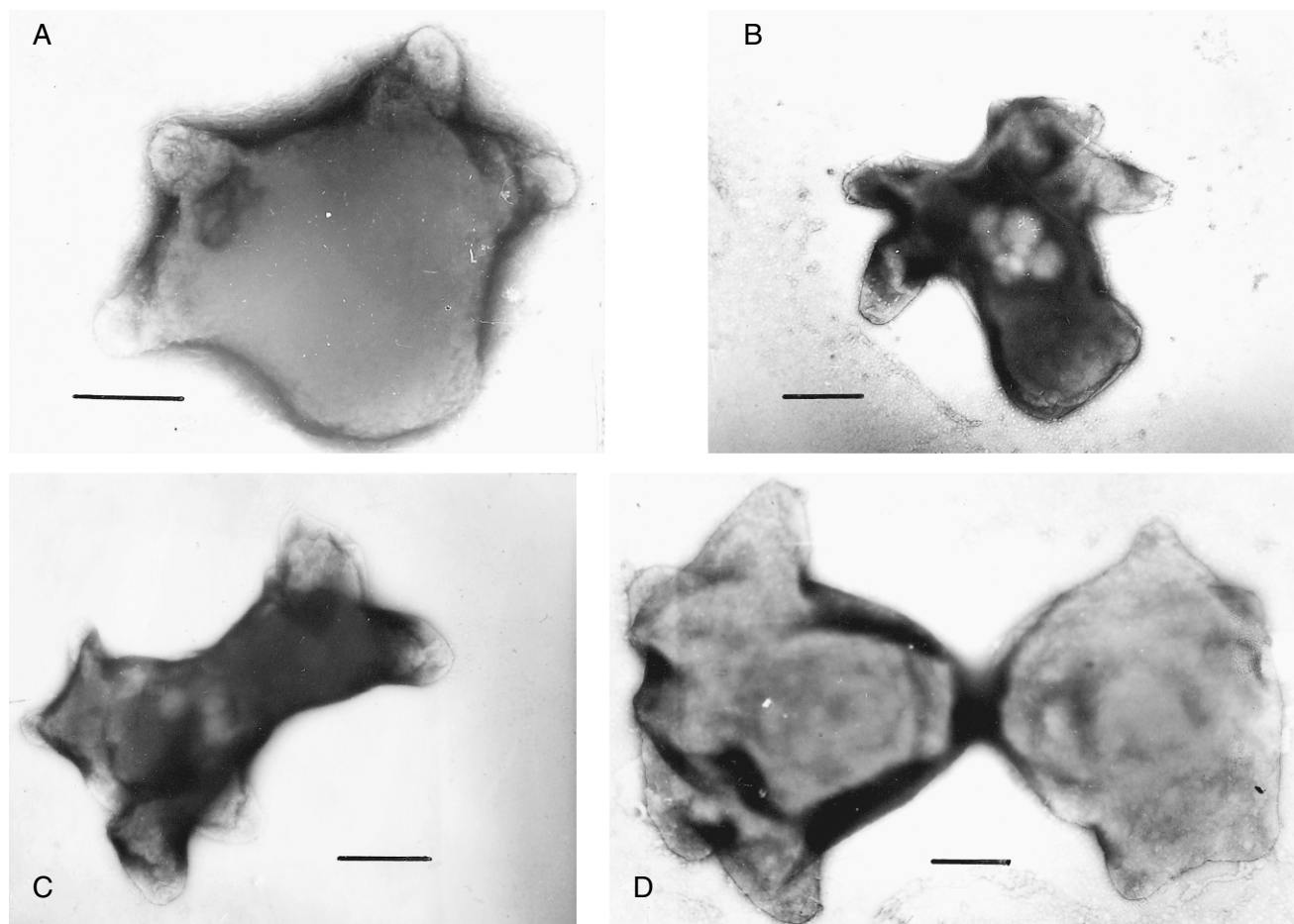


FIGURE BXII.α.212. *L. monachus*. A–D, electron microscopy showing cells in sequential stages of a multiplication. Note prosthecae protruding from two angles of the cell end, the “double ax” shape of the figure formed by the mother and daughter cells (D). Uranylacetate-negative stain. Bar = 0.5 μ m.

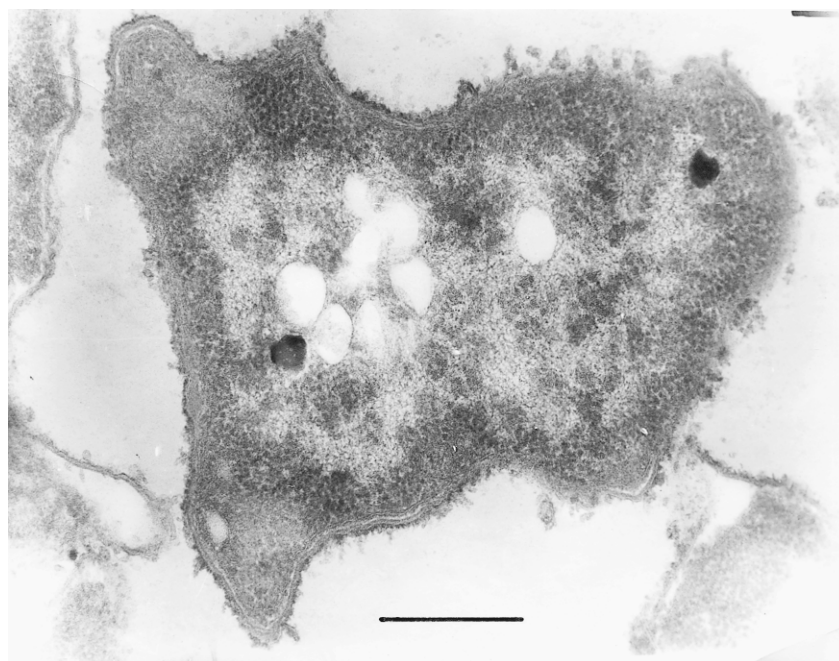


FIGURE BXII.α.213. *L. monachus*. Thin section. Note cytoplasm in prosthecae, granules of poly- β -hydroxybutyrate, and an external microcapsular layer on the outer side of the typical Gram-negative cell wall. Bar = 0.5 μ m.

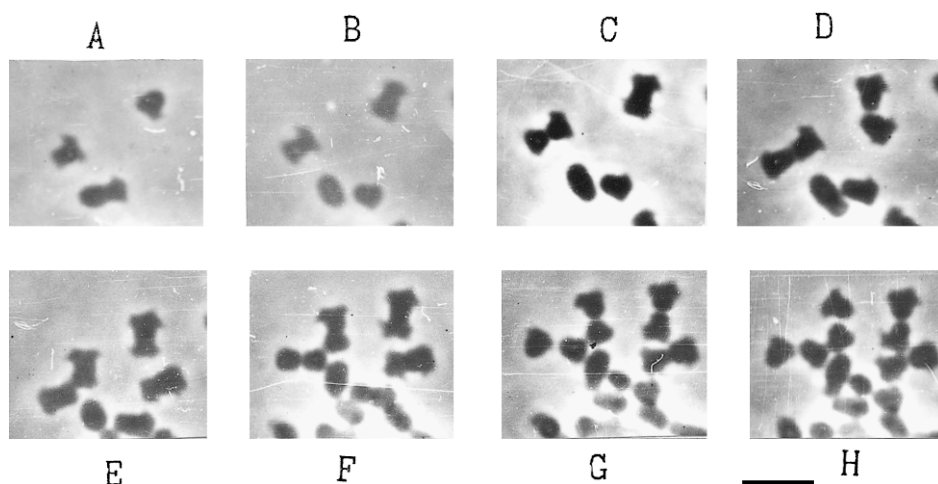


FIGURE BXII.α.214. *L. monachus*. Phase-contrast micrographs. Slide microculture. Hours: A, 0; B, 4; C, 7.5; D, 8.5; E, 11.5; F, 12.5; G, 13; and H, 15. Bar = 5.0 μm .

tose, D-talose, L-fucose, D-mannose, D-galactose, D-tagatose, trehalose, glycerol, mannitol, L-arabitol, adonitol, L-dulcitol, and D-sorbitol. Does not utilize D-lactose, D-cellobiose, D-melibiose, sucrose, D-raffinose, melezitose, starch, inulin, pectin, glycogen, dextrin, or inositol. The medium is acidified during growth on carbohydrates in all instances. Morphology is altered when luxurious growth occurs. Utilization of amino acids, organic acids, and alcohols is limited. Yeast extract above 0.25% inhibits growth; B vitamins are required for growth.

A considerable quantity of poly- β -hydroxybutyrate accumulates in cells immediately after inoculation on all utilized substrates.

Colonies circular, about 2 mm in diameter, colorless or gray, slightly convex, flat, opaque, glistening, smooth, and viscous.

Optimal temperature: 28°C; generation time: 8 h. Isolated from silt of Lake Mustjarv (Estonia).

The mol% G + C of the DNA is: 67.9 (T_m).

Type strain: 42, AUCM B-1479, ATCC 43932, DSM 5896.

Genus XIII. *Methylohabdus* Doronina, Braus-Stromeier, Leisinger and Trotsenko 1996a, 362^{VP} (Effective publication: Doronina, Braus-Stromeier, Leisinger and Trotsenko 1995, 97)

NINA V. DORONINA AND YURI A. TROTSSENKO

Me.thy.lo.rhab'dus. Fr. *méthyle* the methyl radical; G. n. *rhabdos* rod; M.L. masc. n. *Methylohabdus* methyl rod.

Rods 0.4–0.6 \times 1.2–2.5 μm . No endospores. Gram negative. Nonmotile. Multiplication is by fission, with constriction. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Nitrate is reduced to nitrite. Nonpigmented. Growth occurs on nutrient agar and peptone–yeast extract–glucose (PYG) agar. Optimal temperature, 28–34°C; optimal pH, 6.8–7.4. Methyl red and Voges–Proskauer negative. Catalase and urease positive. Oxidase negative. Indole is formed from L-tryptophan in the mineral medium with methanol as a sole carbon and energy source and with KNO₃ as the sole nitrogen source. Ammonium ions inhibit deamination of the tryptophan. Chemoorganotrophic. **Facultatively methylotrophic**; assimilate C₁ compounds (dichloromethane, methanol, and meth-

ylamine) by the isocitrate lyase-negative variant of the serine pathway. The major ubiquinone is Q-10. The cellular fatty acid profile is characterized by the presence of *cis*-vacenic, cyclopropane, and palmitic acids (C_{18:1 ω7}, C_{19:0 cyclo}, and C_{16:0}, respectively). The dominant phospholipids are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, and cardiolipin. Belongs to the *Alphaproteobacteria*.

The mol% G + C of the DNA is: 66.2 (T_m).

Type species: ***Methylohabdus multivorans*** Doronina, Braus-Stromeier, Leisinger and Trotsenko 1996a, 36 (Effective publication: Doronina, Braus-Stromeier, Leisinger and Trotsenko 1995, 97.)

FURTHER DESCRIPTIVE INFORMATION

After division, cells remain connected by a constriction apparently formed by the outer membrane.

Facultative methylotroph. Dichloromethane-grown cells contain an inducible, glutathione-dependent dichloromethane dehalogenase, whereas methanol- or methylamine-grown cells possess the appropriate dehydrogenases. Formaldehyde is further oxidized to formate by glutathione-dependent formaldehyde dehydrogenase. The latter is oxidized to CO₂ by formate dehydrogenases. *Methylohabdus multivorans* uses the isocitrate lyase-negative (icl⁻) variant of the serine pathway and expresses hydroxypyruvate reductase, serine-glyoxylate aminotransferase, and malate lyase. Like other serine pathway methyllobacteria, it has an NADP-dependent isocitrate dehydrogenase.

The primary assimilation of ammonia occurs by reductive amination of α -ketoglutarate.

ENRICHMENT AND ISOLATION PROCEDURES

Methylohabdus multivorans was isolated on dichloromethane agar from an enrichment culture that had been inoculated with a groundwater sample (Doronina et al., 1995).

MAINTENANCE PROCEDURES

The bacteria can be stored in liquid mineral medium with an appropriate C₁ substrate at 4°C for 1 month. For longer-term preservation, freeze-drying can be performed with a protectant (skim milk).

DIFFERENTIATION OF THE GENUS *METHYLORHABDUS* FROM OTHER GENERA

Methylohabdus differs from some serine-pathway methyllobacteria by its formation of constrictions as morphological features (Doronina et al., 1995). *Methylohabdus multivorans* is distinguished from the genus *Methylobacterium* by its absence of pigmentation and by the presence of cyclopropane carboxylic acid in its cellular fatty acid composition. It is distinguished from nonpigmented members of the genus *Aminobacter* by its ability to use methanol, its expression of the isocitrate lyase-negative variant of the serine pathway, and the presence of amine dehydrogenase. It also differs from members of the genus *Hyphomicrobium* by its inability to form hyphae, the presence of the Q-10 quinone system, and the icl⁻ variant of the serine pathway, and its oxidation of methylamine by amine dehydrogenase. It differs from *Methylophil* by its oxidase-negative reaction and the presence of catalase. The major characteristics differentiating the genus *Methylohabdus* from other related genera are summarized in Table BXII.α.180.

TAXONOMIC COMMENTS

In the phylogenetic tree derived from 16S rDNA sequences, the genus *Methylohabdus* forms a distinct branch within the *Alpha-proteobacteria*. The highest degree of relationship is found with *Xanthobacter agilis* (92.6% similarity), *Thiobacillus novellus* (95.7% similarity), and *Ancylobacter aquaticus* (96.2% similarity).

There is less than 10% DNA-DNA similarity with members of *Methylophil*, *Methylobacterium*, *Aminobacter*, *Methylarcula*, and *Hyphomicrobium*.

TABLE BXII.α.180. Major differentiating characteristics of facultative serine pathway methyllobacteria belonging to various genera^a

Characteristics	<i>Methylarcula</i>	<i>Aminobacter</i>	<i>Hyphomicrobium</i>	" <i>Marinosulfonomonas</i> "	<i>Methylobacterium</i>	<i>Methylophil</i>	<i>Methylohabdus</i>	" <i>Methylosulfonomonas</i> "
Morphology (flagella)	—	+	+	+	+	+	—	—
Reproduction by budding	—	—	+	+	—	—	—	—
Reproduction by division	+	+	—	—	+	+	+	+
Hyphae formation	—	—	—	+	—	—	—	—
Oxidase	—	+	+	—	+	—	+	+
Catalase	+	+	+	+	—	+	+	±
Carotenoids	—	+	—	—	—	—	—	—
Reduction of NO ₃ ⁻ to NO ₂ ⁻	+	+	+	+	+	nd	nd	—
<i>Methylamine metabolism:</i>								
Amine dehydrogenase	+	+	—	—	+	nd	nd	—
N-Methylglutamate derivatives	—	+	+	+	—	nd	nd	+
γ-Glutamylmethylamide lyase	—	—	—	—	—	nd	nd	+
Isocitrate lyase	—	—	+	+	—	nd	nd	—
Cyclopropane acid, C _{19:0} cyclo	+	trace	+	+	+	+	+	+
Major ubiquinone	Q-10	Q-10	Q-10	Q-9	Q-10	nd	nd	Q-10
Tolerance to NaCl (%)	2	2.5	2.5	3	2	0.5	3.5	12
Growth at pH 10.0	—	—	—	—	—	—	—	+
Utilization of methanol	+	+	—	+	+	+	+	—
Mol% G + C of DNA	66.2	60–70	62–64	61–65	66–70	61	57	57–61

^aSymbols: +, present; —, absent; nd, not determined.

List of species of the genus Methylohabdus

1. ***Methylohabdus multivorans*** Doronina, Braus-Stromeyer, Leisinger and Trotsenko 1996a, 36^{VP} (Effective publication: Doronina, Braus-Stromeyer, Leisinger and Trotsenko 1995, 97.)

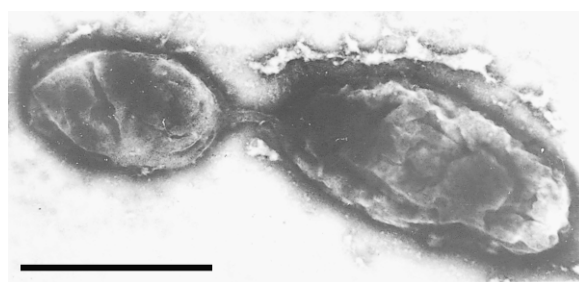
mul.ti'vo.rans. L. adj. *multus* much; L. part. adj. *vorans* devouring, digesting; M.L. part. adj. *multivorans* digesting many compounds.

The characteristics are as described for the genus, with the following additional features. Fig. BXII.α.215 illustrates the morphological features. Colonies on peptone agar medium are white, round with uneven edges, of uniform mucous consistency, and 2–3 mm in diameter. Growth occurs at 10–45°C and at pH 6.0–8.0. No growth occurs in the presence of 3% NaCl. Starch and gelatin are hydrolyzed slowly. Acidification of the medium occurs with glucose, but no gas is formed. Utilizable carbon sources are dichloromethane, methanol, methylamine, L-arabinose, D-xylose, D-glucose, D-mannitol, inositol, glycerol, dulcitol, adonitol, ethanol, isopropanol, acetate, α-ketoglutarate, fumarate, pyruvate, succinate, oxaloacetate, *cis*-aconitate, propionate, L-glutamate, L-alanine, sarcosine, and *N,N*-dimethylglycine. Neither methane nor H₂/O₂/CO₂ supports growth. Methylamine is oxidized to formaldehyde by amine dehydrogenase. The type (and only) strain was isolated from groundwater in Switzerland.

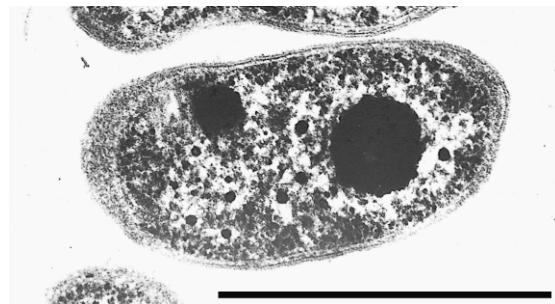
The mol% G + C of the DNA is: 66.2 (*T_m*).

Type strain: DM13, ATCC 51890, VKM B-2030.

GenBank accession number (16S rRNA): AF004845.



A



B

FIGURE BXII.α.215. *Methylohabdus multivorans*. (A) Negatively stained cell. (B) Ultrathin section showing cell-wall structure and polyphosphate granules. Bars = 1 μm.

Genus XIV. ***Pedomicrobium*** Aristovskaya 1961, 957^{AL} emend. Gebers and Beese 1988, 305

PETER HIRSCH AND RAINER GEBERS

Pe.do.mi.cro'bi.um. Gr. n. *pedon* soil; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. neut. n.

Pedomicrobium soil microbe.

Cells oval, spherical, or rod-shaped, 0.4–2.0 × 0.4–2.5 μm. Up to 5 hyphae (i.e., cellular outgrowths or prosthecae of constant diameter with reproductive function) are formed per cell body. Hyphae are 0.15–0.2 μm in diameter. At least one hypha originates laterally; others may appear at the cell poles. Multiplication is by budding at the hyphal tips, with the long axis of the bud arranged perpendicular to the hyphal axis (Figs. BXII.α.216 and BXII.α.217). Mature buds either separate from the hyphae as unflagellated swimmers or remain attached. Extracellular polymers can be stained with ruthenium red and sometimes are visible in India ink mounts as thick capsules around mother cells. Oxidized iron or manganese compounds are deposited on mother cells and later on hyphae. Resting stages are not known. Gram negative, older cells variable. Swarmer cells are motile by a single subpolar or polar flagellum (Fig. BXII.α.218). Other stages of the cell cycle (Fig. BXII.α.219) are nonmotile. Colonies are yellowish or reddish brown to dark brown, due to accumulated iron or manganese oxides. Aerobic. Catalase positive (test is in absence of MnO₂ and at neutral pH). Chemoorganotrophic. Acetate is utilized as a carbon source; most strains also grow on caproate or pyruvate. Protein digests, such as yeast extract, peptone, Casamino acids, and soytone, serve as carbon and/or ni-

trogen sources. Organic nitrogen sources utilized by most strains are glutamate, aspartate, glycine, serine, threonine, and valine. Inorganic nitrogen compounds allow only poor growth of some isolates; nitrate is reduced by most strains. Slow and poor growth occurs on agar media with 0.1–1% fulvic acid iron sesquioxide complexes as sole carbon and nitrogen sources. Vitamin mixtures stimulate growth; lack of vitamins results in pleomorphic cells, which produce large granules of poly-β-hydroxybutyric acid. Growth is inhibited by polymyxin B and neomycin.

A partial oligonucleotide catalogue of the type species, *P. ferrugineum* (IFAM S-1290) has been published (Stackebrandt et al., 1988a).

The mol% G + C of the DNA is: 63–66.8.

Type species: ***Pedomicrobium ferrugineum*** Aristovskaya 1961, 957, emend. Gebers and Beese 1988, 308.

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic treatment The phylogenetic position of *Pedomicrobium ferrugineum* IFAM S-1290^T and various other budding and prosthecae bacteria has been investigated by 16S rRNA cataloguing (Stackebrandt et al., 1988a), revealing an affiliation with *Alphaproteobacteria*. A coherent cluster has been found that em-

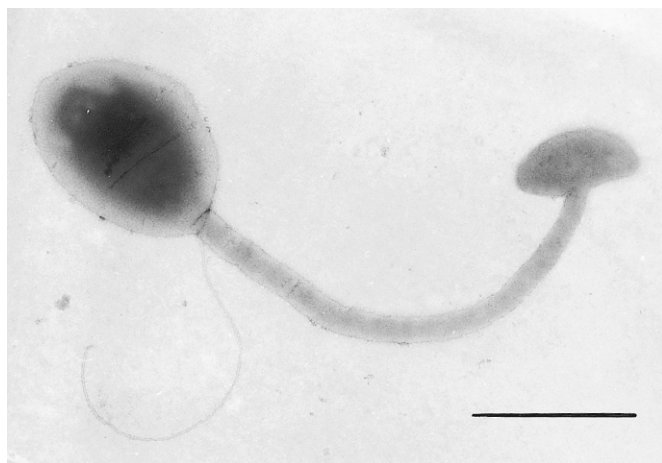


FIGURE BXII.α.216. *Pedomicrobium ferrugineum* IFAM S-1290^T. Transmission electron micrograph showing a mother cell with bud and flagellum. Note the orientation of the bud perpendicular to the hyphal tip. Uranylacetate negatively stained. Bar = 1 μm.

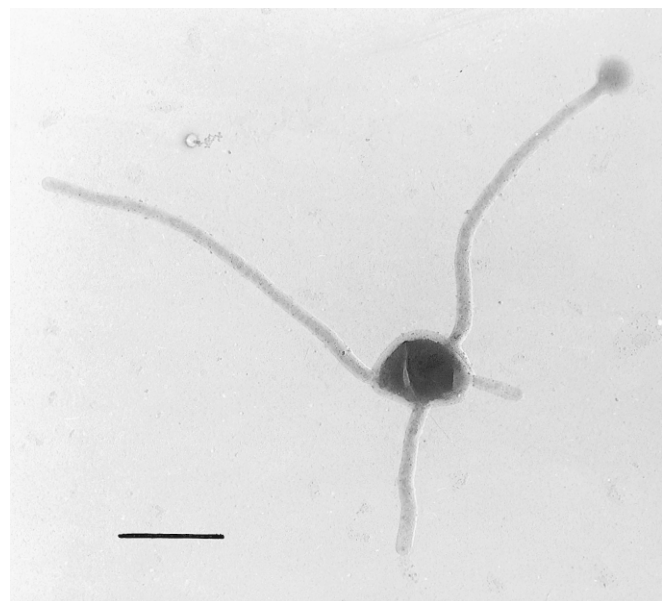


FIGURE BXII.α.217. *Pedomicrobium manganicum* IFAM E-1129^T. Electron micrograph of mother cell with hyphae and young bud. Platinum/carbon-shadowed. Bar = 1 μm.

braces strains of the genera *Hyphomicrobium*, *Filomicrobium*, *Pedomicrobium*, and *Dichotomicrobium*; *Pedomicrobium ferrugineum* and *Filomicrobium fusiforme* are more closely related to some *Hyphomicrobium* strains than they are to each other (Fig. BXII.α.205; see chapter on *Dichotomicrobium*). All of these bacteria share morphological features, such as the formation of prosthecae (hyphae) and reproduction by a budding process. However, the validity of *Pedomicrobium* and *Filomicrobium* as genera separate from *Hyphomicrobium* has been questioned based on morphological traits, which are considered less important for taxonomic separation (Stackebrandt et al., 1988a).

Roggentin and Hirsch (1989), have made an attempt to determine the relationships among 19 *Hyphomicrobium* strains by DNA-rRNA hybrid thermal stability. This study also included *Pe-*

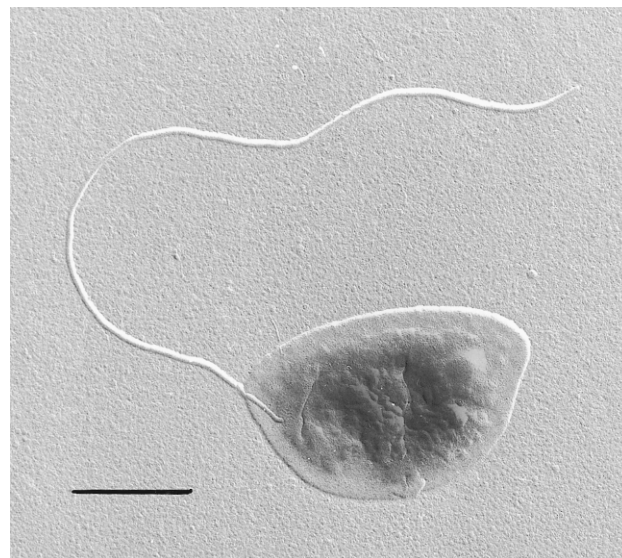


FIGURE BXII.α.218. *P. ferrugineum* IFAM S-1290^T. Electron micrograph showing swarmer cell with subpolar flagellum. Pt/C-shadowed. Bar = 0.5 μm.

domicrobium ferrugineum IFAM S-1290^T, *Hyphomonas* (three strains), *Rhodomicrobium* (one strain), and *Dichotomicrobium* (one strain). The dendrogram derived from average linkage clustering depicts the distant relationships of the *Pedomicrobium* and *Dichotomicrobium* strains to the hyphomicrobia (Fig. BXII.α.220).

Cell morphology Cell shapes of some strains may vary; they can be rod- or spindle-shaped, tetrahedral, pear-, or bean-shaped. Hyphae vary in length according to cultural conditions; true branching occurs. Buds may arise in an intercalary fashion by localized hyphal swelling. Aristovskaya (1961) has occasionally observed direct budding and even cross division of single mother cells, but such observations have not been confirmed by other authors. Deposition of iron or manganese oxides has been observed among all strains studied so far (Aristovskaya, 1961; Khakmun, 1967; Gebers and Hirsch, 1978; Ghiorse and Hirsch, 1979). Many other bacteria with hyphae and buds may (depending on growth conditions) deposit iron or manganese oxides on their cell surfaces (Hirsch, 1968), but the perpendicular positioning of buds at the hyphal tips is a unique characteristic of pedomicrobia.

Chemotaxonomy The quinones, phospholipids, and fatty acids of ten *Pedomicrobium* strains have been studied (Sittig and Hirsch, 1992). All isolates have Q-10 as the respiratory quinone. The phospholipids present are phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and bisphosphatidylglycerol. The fatty acid percentages are as follows: 8–22% normal, 64–89% unsaturated, 1–2% unsaturated/methylbranched, and 0–22% with a cyclopropane ring. Hydroxy fatty acids found in pedomicrobia were C_{14:0} 3OH (82–84%), C_{16:0} 3OH (5–8%), C_{18:1} 3OH (4–5%), and C_{18:0} 3OH (6–8%). A comparative study of pedomicrobia fatty acids (Eckhardt et al., 1979) indicates a strong influence of growth media and culture time on the percentage of octadecenoic acid.

The peptidoglycan of *P. ferrugineum* S-1290^T has been studied following alternating treatments with Pronase P and Na-dodecylsulfate solution with mercaptoethanol (Roggentin and Hirsch, 1982). It has been found to have so little D-alanine that only

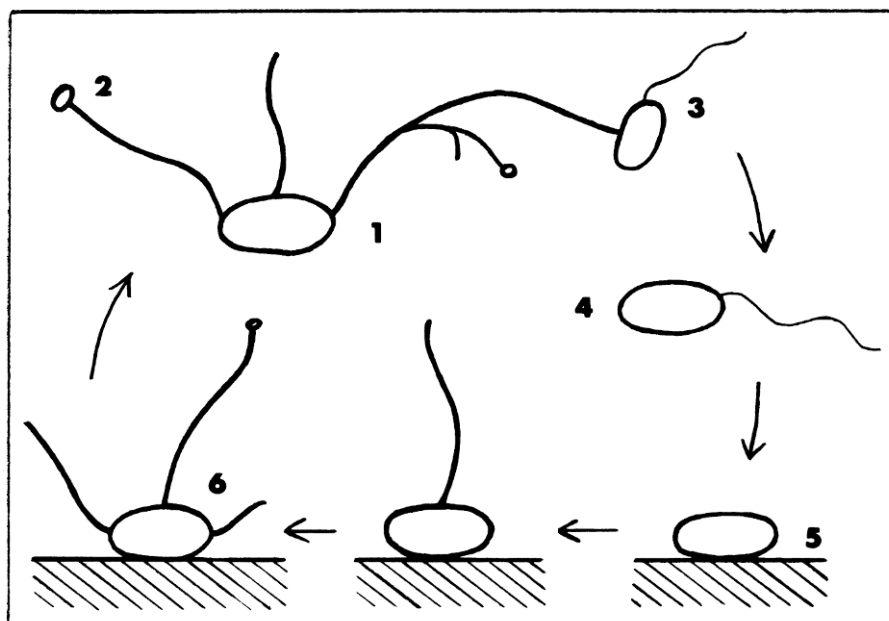


FIGURE BXII.α.219. Life cycle of *Pedomicrobium* species: 1, mother cell with hyphae and buds; 2, young bud; 3, mature bud with flagellum; 4, swarmer cell; 5, young mother cell attached to solid surface; 6, mature mother cell with hyphae and beginning bud formation.

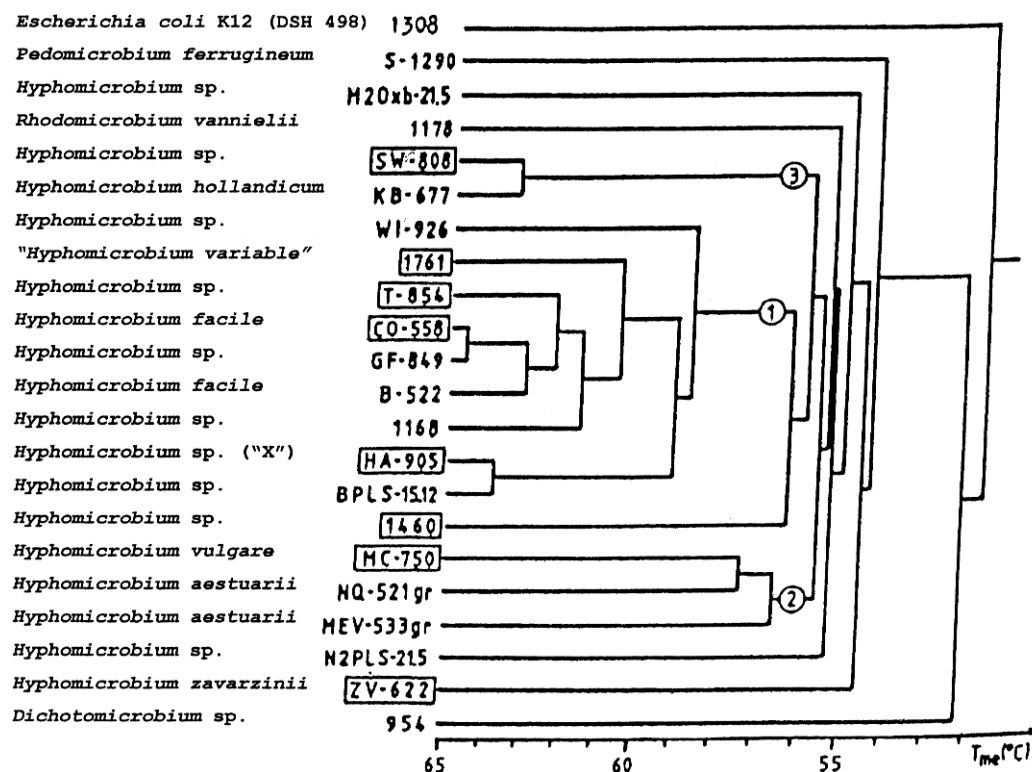


FIGURE BXII.α.220. Dendrogram derived from average linkage clustering showing relationships of *Pedomicrobium ferrugineum* IFAM S-1290^T, *Dichotomicrobium thermohalophilum* IFAM 954^T, *Rhodomicrobium vannielii* IFAM 1178^T (ATCC 17100^T), and various *Hyphomicrobium* strains. The data came from $T_{m(e)}$ values of DNA-rRNA duplexes. Strain numbers in boxes indicate strains that were used as ¹²⁵I labeled references (Roggentin and Hirsch 1989).

every eleventh side chain is cross-linked, a possible explanation as to why the cells can be pleomorphic on certain media.

DNA base compositions of pedomicrobia have been determined by Gebers et al. (1981a and 1985). They range from 64.8 to 65.7 mol% G + C (T_m) for *P. ferrugineum* isolates IFAM S-1290^T, P-1196, Q-1197, R-1198, and T-1130. *P. americanum* strains IFAM G-1381^T, BA-868, and BA-869 have 64.0, 64.7, and 64.4 mol% G + C, respectively (Gebers and Beese, 1988). For *P. manganicum* IFAM E-1129^T, the base ratio is 65.0 mol% G + C, and an unidentified strain (IFAM F-1225) has 64.9 mol% G + C. *Pedomicrobium australicum* strains IFAM ST-1306^T and WD-1255 have 65.0 and 62.8 mol% G + C, respectively.

Fine structure Electron microscopy of ultrathin sections of *Pedomicrobium* isolates IFAM Ba-868 and IFAM Ba-869 reveals a structured surface layer external to the outer membrane layer of the Gram-negative wall. This layer is composed of subunits with approximately 18.5 nm center-to-center spacing (Ghiorse and Hirsch, 1979). Additionally, an electron-dense polymer material is discovered on the mother cell surface of both strains, especially when ruthenium red is included in the fixative (Fig. BXII.α.221). The iron or manganese oxide deposits are present on or within this surface polymer layer. Up to three granules of poly-β-hydroxybutyric acid are stored per cell. Small, dense granules stainable with Loeffler methylene blue are suggestive of polyphosphate storage.

Cultural characteristics All pedomicrobia are aerobic to microaerophilic and grow in the range of 1–21% pO₂ (Gebers and Beese, 1988). On solid media, two types of colonies may develop. Type 1 colonies are round and convex and have even or radially

frayed edges; sometimes they exhibit concentric rings. These colonies have a soft consistency. Type 2 colonies are round, flat and even; they may be crateriform (due to growth into the agar), and the edges are even or radially frayed. The cells in the center of these colonies are often lysed, giving colonies a granular appearance. Type 2 colonies have a cartilaginous consistency and may be removed from the agar intact. Upon spreading on solid media, both colony types give rise to type 1 and 2 colonies. Cultivation with frequent transfers to fresh medium favors large colonies over small ones.

Nutrition and growth conditions Generally, organic acids appear to be the most appropriate carbon sources for *Pedomicrobium* species. Alcohols and carbohydrates are utilized only in some cases (Tables BXII.α.181 and BXII.α.182). Several amino acids serve as nitrogen sources. All strains grow at 20–30°C and in the pH range of 7–9.

Genetics, molecular data Levels of genetic relatedness of nine *Pedomicrobium* isolates with the labeled type strain of *Hyphomicrobium vulgare* (IFAM MC-750^T) have been determined by DNA–DNA hybridization under optimal conditions; Table BXII.α.183 shows homologies of these strains with *H. vulgare* to be only 2–7% (Gebers et al., 1986). The *Pedomicrobium* hybridization data clearly indicate that the four type strains are distinct species.

Genome sizes have been determined for five *Pedomicrobium* strains by DNA renaturation kinetics. The M_r of these strains ranges from 2.81 to 3.0×10^9 Da (Kölbel-Boelke et al., 1985). Plotted against the G + C base ratios, pedomicrobia occupy an area quite distinct from those of other hyphal and budding gen-

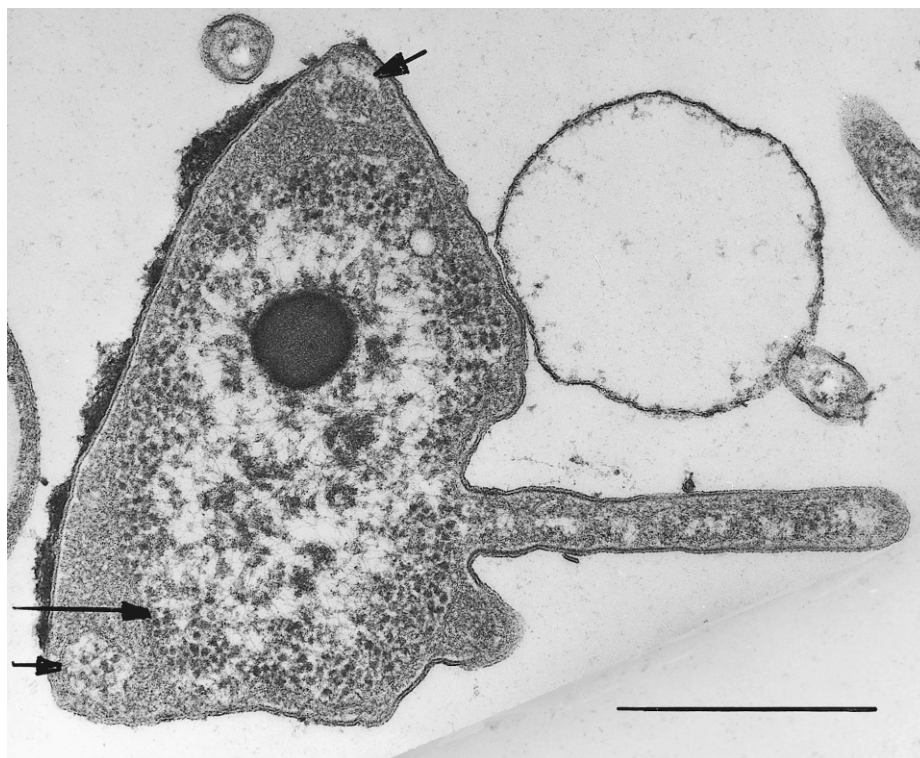


FIGURE BXII.α.221. Thin section of *Pedomicrobium australicum* IFAM ST-1306^T. Note intracytoplasmic membranes (arrows) and dense polyphosphate granule. Deposition of heavy metal oxides occurred primarily on polymer located on the cell surface opposite to the hyphal outgrowth. Bar = 0.5 μm (Reproduced with permission from R. Gebers and M. Beese, *International Journal of Systematic Bacteriology*, 38: 305–315, 1988, ©International Union of Microbiological Societies.)

TABLE BXII.α.181. Characteristics of species of the genus *Pedomicrobium*^a

Characteristic	<i>Pedomicrobium ferrugineum</i>	<i>Pedomicrobium americanum</i>	<i>Pedomicrobium australicum</i>	<i>Pedomicrobium manganicum</i>
Cell size (μm) ^b	0.6–2.0 × 0.6–2.5	1.3 × 1.8	1.2 × 1.8	0.4–0.9 × 0.4–1.5
<i>Flagella:</i>				
Number	1	1	1	1
Attachment	polar or subpolar	subpolar	subpolar	subpolar
Intercalary buds formed occasionally	+	+	+	+
Fe oxide deposited with Fe powder	+	NT	NT	v ^c
Fe oxide deposited with FeS or iron paper clips	+	NT	NT	w
Mn oxide deposited with MnSO ₄ ·H ₂ O	–	+	+	+
Utilization of acetate as carbon source	+	+	+	+
Utilization of ethanol	– ^d	– ^d	– ^d	NT
Growth with phenol	v ^b	– ^d	NT	NT
Growth with propanol	v ^b	– ^d	– ^d	NT
Growth with tartrate	w	NT	NT	NT
Growth with cholesterol or paraffin	w	NT	NT	NT
<i>Utilization of amino acids for growth:</i> ^b				
L-Arginine or L-lysine	+	+	w	NT
L-Isoleucine or L-leucine	+	+	w	NT
L-Aspartate or glycine	+	+	+	NT
L-Glutamate or L-histidine	+	+	+	NT
L-Phenylalanine	+	+	+	NT
L-Proline	+	–	w	NT
L-Serine, L-threonine, or D-valine	+	+	+	NT
L-Tyrosine	+	+	NT	NT
Growth with ammonia as N-source	+	+	+	NT
Growth with 0.05% (w/v) of yeast extract or peptone	+	+	+	w
Growth with urea as N-source	w	NT	NT	NT
Generation time (h) in PSM at 30°C	10	10	11	NT
Storage of PHBA and polyphosphate	+	+	+	+
Vitamin Solution No. 6	Required	Required	NT	Stimulation
Catalase activity	+	D	+	+
Nitrate reduction	+	+	+	NT
Pathogenic for guinea pigs	–	NT	NT	–

^aSymbols: +, 90% or more strains positive; –, 90% or more strains negative; v, variable with growth conditions; D, differs among strains; w, growth weak and slow; NT, not tested or information lacking.

^bData from Gebers (1981) or Gebers and Beese (1988).

^cW.C. Ghiorse, personal communication.

^dInhibition of growth.

era, such as *Hyphomicrobium*, *Hyphomonas*, *Dichotomicrobium* (“genus D”), *Filomicrobium* (“genus F”), or “genus T”.

Bacteriophages Samples from a freshwater lake (Fuhlensee, Kiel, Germany), inoculated with cultures of *Pedomicrobium americanum* IFAM G-1381^T and shaken at 30°C, have resulted in the isolation of phage Pe-60 (C. Gliesche, personal communication). This DNA phage adsorbs to *Pedomicrobium* mother cells as well as to the hyphae (Figs. BXII.α.222 and BXII.α.223). The following IFAM pedomicrobia are lysed by phage Pe-60: G-1381^T, WD-1355, BA-868, BA-869, ST-1306^T, and S-1290^T; however, two *Hyphomicrobium* isolates (B-522 and WI-926) are not susceptible (C. Gliesche, unpublished). Pe-60 has been partially characterized by Majewski (1986). It has a base composition of 57.2 mol% G + C, the molecular weight is 66.86 MDa, and it can be stored

under chloroform or lyophilized. Phage Pe-60 belongs to the *Styloviridae*.

Antibiotic sensitivities The strains of *P. ferrugineum*, *P. americanum*, and *P. australicum* all show high sensitivity to eight antibiotics, but low inhibition by sulfanilamide (Table BXII.α.184). *P. manganicum* is not inhibited by most of these antibiotics (Gebers, 1981; Gebers and Beese, 1988).

Pathogenicity *Pedomicrobium ferrugineum* IFAM S-1290^T and *P. manganicum* IFAM E-1129^T are not pathogenic for mice or guinea pigs within 7 days after intraperitoneal or intravenous injection of up to 10⁸ cells. They are not β-hemolytic and do not utilize mannitol, but all strains cause antibody formation, with agglutination dilution titers reaching 1:2 (Famureva et al., 1983).

TABLE BXII.α.182. Growth of *Pedomicrobium* IFAM strains with selected carbon sources^a

Carbon source	<i>Pedomicrobium ferrugineum</i>					<i>Pedomicrobium americanum</i> ^b			<i>Pedomicrobium australicum</i> ^b		<i>P. manganicum</i> E-1129 ^{Tc}
	S-1290 ^{Tb}	P-1196 ^c	Q-1197 ^c	R-1198 ^c	T-1130 ^c	G-1381 ^T	BA-868	BA-869	ST-1306 ^T	WD-1355	
Formate	—	+	+	—	—	w	+	+	+	w	w
Acetate	+	+	+	+	+	+	+	+	+	+	+
Caproate	+	+	+	+	+	—	—	—	—	w	+
Pyruvate	+	+	+	+	+	+	+	—	—	+	+
D-Lactate	—	w	w	—	—	—	—	—	—	—	+
Citrate	—	NT	NT	NT	NT	—	—	+	—	—	NT
D,L-Malate	w	NT	NT	NT	NT	w	+	+	+	+	NT
Succinate	+	NT	NT	NT	NT	+	w	+	NT	w	NT
D-Glucose	—	+	+	—	—	—	—	—	+	—	w
Lactose	—	NT	NT	NT	NT	—	w	—	+	+	NT
D-Ribose	—	NT	NT	NT	NT	—	—	—	—	—	+
Ethanol	—	NT	NT	NT	NT	—	—	—	—	w	NT
Gluconate	—	NT	NT	NT	NT	w	w	+	+	+	NT
Glycerol	—	NT	NT	NT	NT	w	+	+	+	—	NT
D-Mannitol	w	NT	NT	NT	NT	w	+	—	+	—	NT
Methanol	—	NT	NT	NT	NT	—	—	—	—	w	NT

^aSymbols: +, positive growth; —, no growth; w, growth weak and slow; NT, not tested or information lacking.

^bData from Gebers and Beese (1988).

^cData from Gebers (1981).

TABLE BXII.α.183. Levels of DNA–DNA similarity among *Pedomicrobium* and selected *Hyphomicrobium* strains^a

Taxon	Source of unlabeled DNA		% Similarity with labeled DNA from strain				
	IFAM ^b strain no.	ATCC ^c strain no.	IFAM S-1290 ^T	IFAM G-1381 ^T	IFAM BA-869	IFAM ST-1306 ^T	IFAM E-1129 ^T
<i>Pedomicrobium ferrugineum</i>	S-1290 ^T	33119 ^T	100	16	14	11	14
	Q-1197	33117	99 ^d	17	13	5	12
	T-1130	33120	97 ^d	18	11	10	9
<i>Pedomicrobium manganicum</i>	E-1129 ^T	33121 ^T	13 ^d	10	7	3	100
<i>Pedomicrobium americanum</i>	G-1381 ^T	43612 ^T	NT	100	100	28	8
	BA-868	43613	18	91	94	NT	8
	BA-869	43615	22	82	100	20	6
<i>Pedomicrobium australicum</i>	ST-1306 ^T	43611 ^T	18	25	26	100	8
	WD-1355	43614	17	25	29	87	8
<i>Hyphomicrobium vulgare</i>	MC-750 ^T	27500 ^T	7 ^d /(4) ^c	NT	2/(4)	(5)	2/(5)
<i>Hyphomicrobium</i> sp.	T-854 (T37)		5	NT	2	NT	1

^aData from Gebers and Beese (1988). Similarity data of at least two reactions, corrected for background values obtained with self-reassociation controls.

^bIFAM, Institut für Allgemeine Mikrobiologie, Universität Kiel, Germany.

^cATCC, American Type Culture Collection, Rockville, Md., USA.

^dData from Gebers et al. (1981b).

^eData in parentheses are values for reciprocal reactions (Gebers et al., 1986).

Ecology *Pedomicrobium* species are widely distributed in podzolic and other soils, in freshwater lakes, ponds, and brooks, in iron springs, and in seawater; they are ubiquitous (Table BXII.α.185).

ENRICHMENT AND ISOLATION PROCEDURES

Isolation of *P. ferrugineum* and *P. manganicum* from soil, especially from podzolic soil, can be achieved by Aristovskaya’s procedure (Aristovskaya, 1961) as described by Gebers and Hirsch (1978, 1979). Podzolic soil samples are suspended by repeatedly shaking in 0.85% (w/v) saline solution and are streaked onto humic gel agar¹. Since fulvic acids serve as sole carbon and nitrogen sources, this medium is rather selective, but allows only slow growth of *Pedomicrobium* species. After 3–12 weeks incubation at 20 or 30°C

in the dark, *Pedomicrobium*-containing colonies of the agar plate “enrichment” may be recognized by their yellowish brown to dark brown color, due to accumulation of iron and/or manganese oxides. Identification of iron-depositing colonies by the Prussian blue reaction is difficult because humic gel usually contains Fe (III) and thus stains intensely. Screening for manganese-depositing colonies, however, is facilitated by flooding the enrichment plate with leuko-berbelin blue I or leuko-crystal violet. Since *Pedomicrobium*-containing colonies are strongly coherent, spreading onto agar plates is enhanced by sterile grinding of the inoculum in a drop of saline solution. *Pedomicrobia* obtained with this agar plate enrichment procedure grow, albeit slowly, on humic gel agar without any other additions.

For final purification streaks, humic gel agar is supplemented with a vitamin solution². This is necessary because *Pedomicrobium*

1. Humic gel agar: Humic gel (fulvic acids complexed with metal sesquioxides) is prepared by hydrochloric acid extraction from (podzolic) humus soil (Ponomareva, 1964; Gebers and Hirsch, 1978). 5 g (wet weight) humic gel and 18 g agar (Difco) are suspended in 1000 ml of distilled water and autoclaved; the mixture is poured as thick layers into Petri plates; the final pH is 5–6.

2. Vitamin solution (Van Ert and Staley, 1971) consists of: 2 mg biotin, 2 mg folic acid, 5 mg thiamin·HCl, 5 mg calcium pantothenate, 0.1 mg cyanocobalamin, 5 mg riboflavin, 5 mg nicotinamide, 5 mg *p*-aminobenzoic acid, 10 mg pyridoxin·HCl, and 1000 ml distilled water.

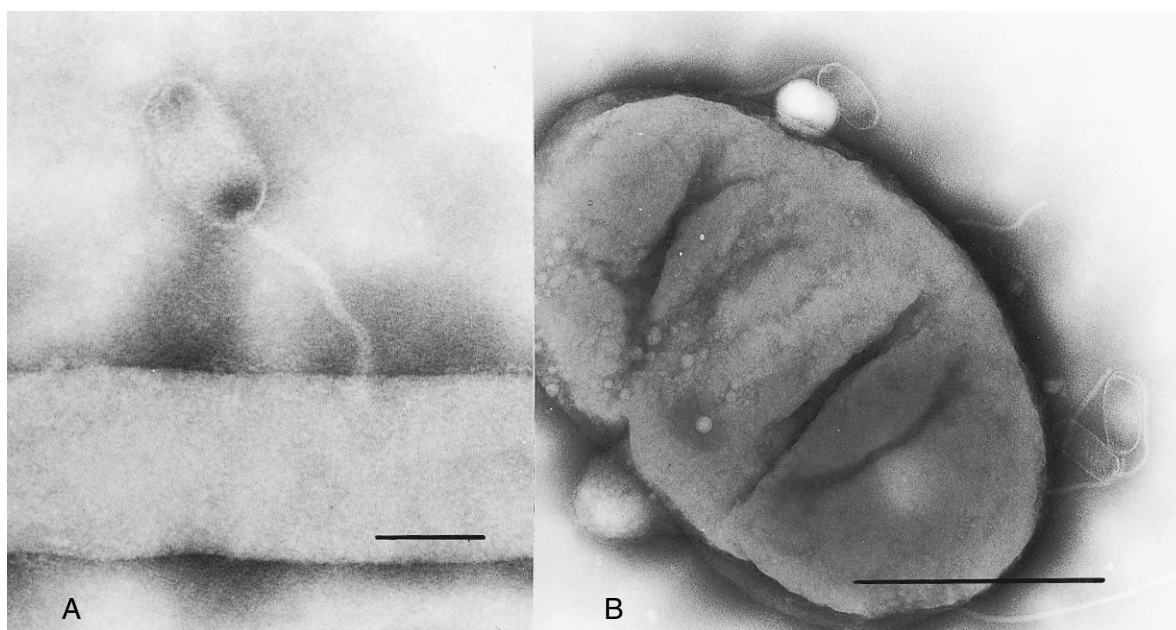


FIGURE BXII.α.222. Adsorption of phage Pe-60 of *Pedomicrobium americanum* IFAM G-1381^T to a hypha (A) or mother cell (B). Negatively stained with 1% uranylacetate. Bars = 0.1 μ m (A) and 0.5 μ m (B). (Courtesy of C. Gliesche and D.M. Majewski, unpublished.)

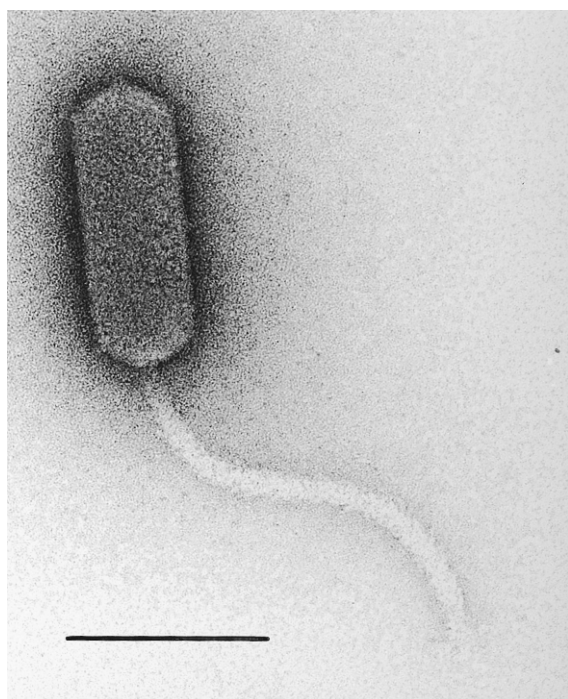


FIGURE BXII.α.223. Phage Pe-60 (Styloviridae) from *Pedomicrobium americanum* IFAM G-1381^T. Electron micrograph on carbon-coated mica negatively stained with 1% uranylacetate. Bar = 0.1 μ m. (Courtesy of C. Gliesche and D.M. Majewski, unpublished.)

cells have an irregular, pleomorphic appearance when grown without vitamins. *Pedomicrobia* also grow at pH 7.0 in synthetic media, which contain (besides some nutrients) either 400 mg/50 ml iron powder, an iron paper clip (400 mg), or 630 mg FeS (Gebers and Hirsch, 1979). *Pedomicrobium* standard medium

(PSM)³ supplemented with 0.015% (w/v) Actidione (Roth, Germany) and 1.8% agar may serve as an alternative medium for enrichment and isolation of pedomicrobia. Actidione inhibits many fungi, but this medium also allows growth of contaminating bacteria; therefore, the selectivity for *Pedomicrobium* species is low.

Aquatic strains of *Pedomicrobium* spp. have been enriched by Staley et al. (1980) as follows: water samples are diluted in a series of test tubes with peptone medium DPM⁴ up to the 10⁻⁹ dilution. Inoculated tubes are incubated at 20–30°C until surface pellicles develop in the highest dilution tubes. For isolation of pedomicrobia, the pellicles are streaked onto DPM solidified with 1.8% agar. A combination of growth on indicator medium PC and micromanipulation has enabled Sly and Arunpairojana (1987) to isolate manganese-oxidizing pedomicrobia from freshwater. The PC medium employed here contains (w/v): 0.005% Difco yeast extract, 0.002% MnSO₄·4H₂O, 2% Bacto agar, and 50 μ g/ml of Actidione (Upjohn, USA).

MAINTENANCE PROCEDURES

Pedomicrobium species grown on PSM agar slants survive for more than 2 years in tightly sealed screw-capped tubes at 20–30°C. For longer preservation, 1 part of an exponentially growing liquid

3. PSM consists of 10 mM sodium acetate, 0.5 g yeast extract (Difco), 1 ml "metals 44" (see below), 10 ml vitamin solution, and distilled water to 1000 ml. Adjust the pH to 9.0; final pH after autoclaving: 7.0. "Metals 44" (Cohen-Bazire et al., 1957) consists of: 125 mg EDTA, 547.5 mg ZnSO₄·7H₂O, 250 mg FeSO₄·7H₂O, 77 mg MnSO₄·H₂O, 19.6 mg CuSO₄·5H₂O, 12.4 mg Co(NO₃)₂·6H₂O, 8.85 mg Na₂B₄O₄·10H₂O, and 50 ml distilled water. Adjust pH to 6.8.

4. DPM consists of 0.1 g peptone (Difco), 20 ml Hutner's modified salts solution (see below), 10 ml vitamin solution, and distilled water up to 1000 ml. Hutner's modified salts solution (Cohen-Bazire et al., 1957) contains (per liter, w/v): 10 g nitrilotriacetic acid, 29.7 g MgSO₄·7H₂O, 3.3 g CaCl₂·2H₂O, 12.7 mg NaMoO₄·2H₂O, 99.0 mg FeSO₄·H₂O, and 50 ml "metals 44". Distilled water is added after pH adjustment. The nitrilotriacetic acid is first neutralized with potassium hydroxide. Then, the remaining ingredients are added before the pH is adjusted to 7.2 with KOH and H₂SO₄. Finally, distilled water is added to make 1 liter of solution.

TABLE BXII.α.184. Growth inhibition of IFAM *Pedomicrobium* strains by antibiotics (100 µg/ml)

Antibiotic	<i>Pedomicrobium ferrugineum</i> S-1290 ^T	<i>Pedomicrobium americanum</i>			<i>Pedomicrobium australicum</i>		<i>Pedomicrobium manganicum</i> E-1129 ^T
		G-1381 ^T	BA-868	BA-869	ST-1306 ^T	WD-1355	
<i>Liquid cultures:</i> ^a							
Ampicillin	82	95	93	91	91	91	—
Penicillin G	87	93	97	90	97	95	NT
Cephalothin	+ ^b	77	68	+ ^b	+ ^b	84	—
Cycloserine	82	95	96	92	94	95	+ ^c
Polymyxin B	+ ^b	+ ^b	+ ^b	+ ^b	+ ^b	+ ^b	+ ^c
Neomycin	84	84	82	84	83	95	+ + ^c
Chloramphenicol	79	92	98	88	98	82	—
Streptomycin	+ ^b	+ ^b	+ ^b	+ ^b	+ ^b	+ ^b	NT
Sulfanilamide	16	3	22	47	8	33	—
<i>Plate diffusion tests:</i> ^c							
Nalidixic acid	—	NT	NT	NT	NT	NT	—
Rifampin	+ + ^c	NT	NT	NT	NT	NT	—
Tetracycline	+ + ^c	NT	NT	NT	NT	NT	—
Gentamicin	—	NT	NT	NT	NT	NT	+ + ^c
Nitrofurazon	—	NT	NT	NT	NT	NT	—

^aData from Gebers and Beese (1988). Percent inhibition of protein formation as compared to untreated cultures. Symbols: +, strong inhibition (~60–100%); + +, inhibition zone >10 mm; +, inhibition zone 1–10 mm; —, no inhibition; NT, not tested.

^bInhibition estimated by visual comparison of culture turbidities.

^cData from Gebers 1981, determined by plate diffusion tests on PSM agar.

culture is freeze-dried at –55°C; addition of milk can be omitted. Viability of these lyophils needs to be tested frequently, and counting of the percentage of survivors is necessary. Medium PSM, either autoclaved or sterile-filtered, is recommended for the revival of such lyophilized cultures and for subculturing. Freeze-dried cultures are stored at 4°C and should not be kept for more than 2–3 years. Storage in liquid nitrogen may be required.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Testing for iron and manganese oxide accumulation from fulvic acid sesquioxide complexes is impractical because these humic substances are available solely from podzolic soils, which occur only in certain regions on earth. Therefore, it is recommended that instead of fulvic acid complexes embedded in agar media, elemental iron, FeS, or MnSO₄, be employed. The specificity of the test is reduced, however, since autoxidation of elemental iron and pyrite occurs at physiological pH, and various bacteria are capable of accumulating the oxidized products. The presence of ferric iron is indicated by a blue color around cells or colonies when a solution of 2% K₄[Fe(CN)₆], acidified with HCl, is added (Prussian blue reaction). The presence of manganese (IV) is demonstrated by a blue color reaction with a 0.4% (w/v) solution of leuko-berbelin blue I (Krumbein and Altmann, 1973) or with Feigl's reagent (1% [w/v] benzidinehydrochloride in a 7% [v/v] acetic acid solution). In contrast to Feigl's reagent, leuko-berbelin blue I does not inhibit viability of the cells.

Methods used for cell wall disintegration, DNA extraction, and purification have been described by Gebers et al. (1985). Shearing of DNA and radioactive labeling have been described by Gebers et al. (1981b, 1986). DNA reassociation procedures, S1 nuclease treatment, and specific conditions for hybridization reactions are given by Gebers and Beese (1988).

DIFFERENTIATION OF THE GENUS *PEDOMICROBIUM* FROM OTHER GENERA

Table BXII.α.186 provides primary characteristics that can be used to differentiate the genus *Pedomicrobium* from morphologically similar taxa. The most important property of pedomicrobia

is the bud elongation perpendicular to the hyphal long axis; deposition of oxidized iron or manganese on the cell surface is not restricted to *Pedomicrobium* species, but either is often dependent on the production of polyanionic surface polymers (Ghiorse and Hirsch, 1978a).

TAXONOMIC COMMENTS

Originally, five species of *Pedomicrobium* were described: *P. ferrugineum*, *P. manganicum* (Aristovskaya, 1961), "*P. podsolicum*" (Aristovskaya, 1963), *P. americanum*, and *P. australicum* (Gebers and Beese, 1988). None of several *Pedomicrobium*-like isolates known fit the description of "*P. podsolicum*", and cultures of this species are not available. In addition, it is not mentioned in the Approved Lists of Bacterial Names.

Some manganese oxide-depositing bacteria discussed in the literature have been considered to be pedomicrobia, either because of their growth on organomineral complexes of fulvic acids and/or because of the presence of a large number of hyphae on the mother cells. Khakmun (1967) has observed such bacteria in soils of Sakhalin and named them *Pedomicrobium manganicum* biovar sachalinicum because of their more oval cell shape. Neither a pure culture nor a formal description of these is available. A micrograph published shows *Hyphomicrobium*-like, possibly budding bacteria, but lacks any indication of a perpendicular bud location on the hyphal tips. Epicellular iron-depositing aquatic bacteria studied by Hirsch (1968) or Hirsch and Rheinheimer (1968) were originally considered to represent pedomicrobia, but these bacteria also resemble hyphomicrobia and lack the perpendicular bud orientation on the hyphal tips. A bacterial isolate (strain T37) with manganese oxide deposition on the cell surface has been obtained from manganese oxide sludge on the inside of hydroelectric pipelines (Tyler and Marshall, 1967). This organism resembles hyphomicrobia morphologically, grows with methanol, and does not show the characteristic positioning of the buds. After subculturing, it eventually loses the ability to oxidize and deposit manganese, but instead oxidizes iron (Ghiorse and Hirsch, 1978b).

Real pedomicrobia have been observed by Kutuzova et al. (1972) after exposing slit peloscopes in ooze-containing vessels.

TABLE BXII.α.185. Differential characteristics of *Pedomicrobium* species^a

Characteristic	<i>Pedomicrobium ferrugineum</i> IFAM S-1290	<i>Pedomicrobium americanum</i> IFAM G-1381	<i>Pedomicrobium australicum</i> IFAM ST-1306	<i>Pedomicrobium manganicum</i> IFAM E-1129
<i>Cell shape:</i>				
Coccioid	+			+
Oval	+	+	+	+
Rods	+	+	+	+
Spindles		+	+	
Tetrahedral	+	+	+	
Cell size (μm)	0.6–2.0 × 0.6–2.5 ^b	1.3 × 1.8 ^b	1.2 × 1.8 ^b	0.4–0.9 × 0.4–1.5 ^c
<i>Hyphal origin:</i>				
Laterally	+	+	+	+
Polarly	+	+	+	+
Subpolarly	+			
Number of hyphae per mother cell	1–3	1–3	1–3	1–5
Deposit Fe ³⁺ (growth with fulvic acid sesquioxide complexes)	+	+	+	–
Deposit Fe ³⁺ (growth in presence of Fe powder)	+	NT	NT	+ ^d
Deposit Fe ³⁺ (growth on PSM + FeSO ₄ ·7H ₂ O)	NT	+	+	NT
Deposit Mn oxide (growth on PSM + MnSO ₄ ·H ₂ O)	–	+	+	+
Intracytoplasmic membranes may be formed	–	v	v	v
Growth with formate	–	w ^b	+	w
Growth with pyruvate	+	+	–	+
Growth with D,L-malate or D-mannitol	w ^b	w ^b	+	NT
Growth with lactose	–	–	+	NT
Growth with D-glucose	–	–	+	w
Growth with L-proline	+	–	w	NT
Utilization of NaNO ₃ as N-source for growth	w	–	–	NT
Temperature range for growth (°C)	10 ^c –43	15–41	15–36	NT
Temperature optimum for growth (°C)	29–30	32–38	29–32	30
<i>Dependence on Vitamin Solution No.6 (Van Ert and Staley, 1971):</i>				
Required	+	+		
Stimulated				+
Growth inhibition by ampicillin, cephalothin, or chloramphenicol	+	+	+	–
<i>Habitat (source):</i>				
Podsollic soil, Germany	+			
Freshwater pond and puddle, USA		+		
Fresh water, N. S. W., Australia			+	
Quartzite rock pool, France				+

^aSymbols: +, positive; –, negative; w, weak or slow reaction; v, variable with growth conditions; NT, not tested or information lacking.^bData from Gebers and Beese (1988).^cData from Gebers (1981).^dW.C. Ghiorse (personal communication).

One of her electron micrographs labeled “*Pedomicrobium*-type organism” (Fig. 1e) shows a tetrahedral mother cell, but with a straight bud attachment to the hypha, somewhat similar to that of *Dichotomicrobium* spp. However, Fig. 2g in that reference shows a bacterium with the morphology of a *Pedomicrobium* sp.; pure cultures have not been isolated.

Recently, Sly et al. (1988) have isolated eight strains of manganese-oxidizing pedomicrobia from “dirty drinking water” and

biofilm samplings of southeast Queensland, Australia. Pure cultures have been obtained by micromanipulation and inoculation of PC agar, which contains (w/v): 0.005% Difco yeast extract, 0.002% MnSO₄·4H₂O, and 2% Bacto agar (Sly and Arunpairojana, 1987). To control fungal growth, 50 μg/ml of Actidione (Pfizer, USA) were added. The morphology of these new isolates is thought to correspond closely to that of the *P. manganicum* type strain (IFAM E-1129^T), although cells and mature swarmer

TABLE BXII.α.186. Differential characteristics of the genus *Pedomicrobium* and other morphologically similar taxa^a

Characteristic	<i>Pedomicrobium</i>	<i>Hyphomicrobium</i>	<i>Filomicrobium</i> ^b	<i>Hyphomonas</i>	<i>Hirschia</i> ^c	<i>Dichotomicrobium</i>	<i>Rhodomicrobium</i>
<i>Cell shape:</i>							
Bean-shaped	+	+		+	+		+
Coccoid	+			+	+	+	+
Cubical						+	
Fusiform			+				+
Ovoid	+	+		+	+		+
Pear-shaped		+		+	+		+
Rod-shaped	+	+		+	+		+
Spindles		+					+
Tetrahedral	+					+	+
<i>Origin of hyphae:</i>							
Lateral	+					+	
Polar	+	+	+	+	+	+	+
Number of hyphae per mother cell	1–5	1–4	2–3	1–2	1–2	1–4	1–2
Hyphae with septa	–	–	–	–	–	–	+
Bud: long axis perpendicular to hypha	+	–	–	–	–	–	–
Accumulate Fe or Mn oxides on surface	+	v ^d	–	–	–	–	–
<i>Utilization of:</i>							
C ₁ compounds	D	+	–	–	–	NT	v
Formate	D	D	–	–	–	NT	D
Acetate	+	D	+	–	+	+	+
<i>Major quinone component:</i>							
Q-9		+	+				
Q-10	+			+	+	+	+
Q-11				+			
Photosynthetic pigments	–	–	–	–	–	–	+
<i>Nitrogen source:</i>							
NaNO ₂	–	D	NT	– ^e	NT	–	NT
NaNO ₃	D	+	+	–	+	–	D
NH ₄	+ ^f	+	+	–	+	–	+
Grow in presence of 3.5% (w/v) NaCl	– ^g	D	+	+	+	+	NT
Genome size M_r ($\times 10^9$) ^h	2.81–3.43	2.13–2.73	2.03	1.67–2.00	NT	1.73	NT
Mol% G + C of DNA (T_m)	63–66 ^{ij}	59–65	62	57–62	45–47	62–64	62–64 (Bd)

^aSymbols: +, 90% or more strains positive; –, 90% or more strains negative; v, variable with growth conditions; D, differs among strains; NT, not tested or information lacking.

^bData from Schlesner (1987).

^cData from Schlesner et al. (1990).

^dData from Hirsch (1968).

^eData from Gebers et al. (1984).

^fData from Gebers and Beese (1988).

^gSurvival but no growth (Gebers, 1989).

^hData from Kölbel-Boelke et al. (1985).

ⁱData from Gebers et al. (1985).

^jData from Gebers et al. (1981a).

cells of the new isolates are slightly smaller: $0.4\text{--}0.6 \times 0.8\text{--}1.2 \mu\text{m}$ and $0.4 \times 0.6 \mu\text{m}$, respectively, as compared to IFAM E-1129^T, whose cells and swarmer cells are $0.4\text{--}0.9 \times 0.4\text{--}1.5 \mu\text{m}$ and $0.9 \times 1.5 \mu\text{m}$, respectively. The buds develop at hyphal tips in a lateral (perpendicular) as well as a polar orientation; mother cells have no more than four hyphae. There are also some physiological differences from the type strain. The eight new isolates fail to grow with acetate or glutamate as the sole carbon source; Vitamin Solution no. 6 does not stimulate growth, and besides manganese they oxidize iron, albeit much less rapidly. Their DNA base ratio range is 65.6–66.6 mol% G + C.

Sly et al. (1988) considered these eight isolates to be new strains of *P. manganicum*, which may have appeared justifiable in view of the fact that this species was based on only one isolate and thus its variability was not yet known. The observation of lateral, as well as polar, positions of buds on hyphal tips raises questions about an important differentiating characteristic of the genus. This aspect needs further investigation.

The phylogenetic relationships of six *Pedomicrobium* strains and some other hyphal and budding *Alphaproteobacteria* have been recently investigated by 16S rRNA sequence analysis (Cox and Sly, 1997, Fig. 1). Unfortunately, *Pedomicrobium australicum* strains IFAM ST-1306^T and IFAM WD-1355 could not be revived from lyophilized cultures (P. Hirsch, personal communication) and thus, DNA of these strains had to be extracted from the lyophils. The other pedomicrobia studied came as viable cultures from the Australian Culture Collection (ACM). The whole 16S rRNA genes were sequenced in both the forward and reverse directions and the sequences aligned manually with sequences of *Hyphomicrobium vulgare*, *Hyphomonas jannaschiana*, *Hirschia baltica*, *Rhodomicrobium vannielii*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Desulfovibrio desulfuricans*, and *Campylobacter jejuni*. The pedomicrobia cluster coherently and separately from the other genera of budding *Alphaproteobacteria* and have sequence similarities of 96.2–99.9%. Within this cluster, *P. manganicum* IFAM E-1129^T is the most distantly related strain to the other pedomicrobia. Strain

ACM 3067, one of the eight manganese-oxidizing isolates from "dirty drinking water" (Sly et al., 1988), is highly related to *P. americanum* IFAM G-1381^T, and the two *P. australicum* strains (IFAM ST-1306^T and IFAM WD-1355) are almost identical, with a sequence similarity of 99.9%. Also highly related, with sequence similarities greater than 99%, are *P. americanum*, the two *P. australicum* strains, and the isolate ACM 3067. Because of their data and some phenotypic differences, Cox and Sly (1997) have questioned the taxonomic validity of *Pedomicrobium australicum*, but suggested retaining *P. americanum*. Differences between these two species in the utilization of carbon sources (Table BXII.α.182) and temperature optima (Table BXII.α.185) may well be an expression of their variability, as similar differences also exist among the three isolates of *P. americanum*. However, since the low DNA-DNA similarity data (Table BXII.α.183) clearly indicate differences on the species level, more phenotypic information and

further studies of viable cultures (as well as a neotype culture) of *P. australicum* are needed to solve this problem.

ACKNOWLEDGMENTS

We gratefully acknowledge provision of *Pedomicrobium*-like strains by J.A. Babinchak, E. Dale, W.C. Ghiorse, and J.T. Staley. Skillful technical assistance was rendered by M. Beese, B. Hoffmann, J. Kock, K. Lutter-Mohr, and U. Wehmeyer. We are also grateful for help and discussions with T.V. Aristovskaya (St. Petersburg).

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DIFFERENTIATION OF THE SPECIES OF THE GENUS *PEDOMICROBIUM*

The differential characteristics of the species of *Pedomicrobium* are listed in Table BXII.α.185. Other descriptive characteristics

are summarized in Tables BXII.α.181, BXII.α.182, BXII.α.183, and BXII.α.184.

List of species of the genus *Pedomicrobium*

1. ***Pedomicrobium ferrugineum*** Aristovskaya 1961, 957^{AL} emend. Gebers and Beese 1988, 308.
fer.ru.gi'ne.um. M.L. neut. adj. *ferrugineum* of iron color.

Cells oval, spherical, rod-shaped, tetrahedral, or bean-shaped, 0.6–2.0 × 0.6–2.5 μm. Oxidized iron, but not manganese, is deposited on mother cells and occasionally later on hyphae. Swarmer cells with one polar or subpolar flagellum. Morphology of colonies as given for the genus. Further morphological and physiological characteristics are listed in Tables BXII.α.181, BXII.α.182, BXII.α.183, BXII.α.184, BXII.α.185, and BXII.α.186.

Heterotrophic, aerobic to microaerophilic. The temperature for optimal growth is 29–30°C, and the temperature range is 10–40°C. Optimal pH for growth: 9.0; the pH range is 3.5–10.0. Growth occurs in the presence of up to 0.1% (w/v) NaCl; no growth, but survival, has been observed in the presence of 1–5% NaCl. Higher concentrations of NaCl are bacteriocidal. Requires cyanocobalamine (1 μg/l) for growth.

Isolated from podzolic soils in northern Germany.

The mol% G + C of the DNA is: 64.5–66.8 (*T_m*, Bd).

Type strain: IFAM S-1290, ATCC 33119, DSM 1540.

GenBank accession number (16S rRNA): X97690.

Additional Remarks: Other strains include IFAM P-1196 (ATCC 33116), IFAM Q-1197 (ATCC 33117), IFAM R-1198 (ATCC 33118), and IFAM T-1130 (ATCC 33120).

2. ***Pedomicrobium americanum*** Gebers and Beese 1988, 310^{VP} *a.me.ri.ca'num.* M.L. neut. adj. *americanum* obtained from America.

Cells oval, tetrahedral, short rods, bean-, or spindle-shaped, up to 1.3 × 1.8 μm (Fig. BXII.α.224). Mother cells with 1–3 hyphae originating laterally or polarly. Bud attachment perpendicular to the hyphal tip; intercalary budding occurs. Swarmer cells with one subpolar flagellum. Extracellular polyanionic polymer located primarily on cell surfaces opposite lateral hyphae, with incrustations of iron or manganese oxides (depending on growth conditions). Intracytoplasmic membranes have been observed.

The temperature optimum for growth is 32–38°C; the range is 15–41°C. The pH optimum for growth in HEPES-buffered media is 7.6–8.4. Growth on carbon sources varies with the strains; all isolates grow on acetate, but none utilizes caproate, D-lactate, D-glucose, D-ribose, methanol, or ethanol. Strain IFAM BA-869 also grows on citrate, formate, gluconate, glycerol, D,L-malate, propanol, and succinate. Good growth occurs with (w/v) 0.05% yeast extract or 0.025% yeast extract plus 0.025% peptone. Nitrate does not



FIGURE BXII.α.224. *Pedomicrobium americanum* IFAM G-1381^T, Pt/C-shaded. Note orientation of buds. Bar = 2 μm. (Electron micrograph courtesy of H. Völker, Kiel.)

serve as a sole nitrogen source, but ammonia and several amino acids support growth (Table BXII.α.181). Cyanocobalamin (1 µg/l) is required for growth of the type strain, IFAM G-1381^T. Growth of strain IFAM BA-868 is stimulated by 0.1% NaCl. Catalase is produced, except by strain IFAM BA-869. Nitrate is reduced by all strains. The three isolates are inhibited by several antibiotics (Table BXII.α.184), but the type strain is resistant to sulfanilamide.

Habitat: bogwater and freshwater puddles in North America.

The mol% G + C of the DNA is: 64–65 (T_m).

Type strain: ACM 3090, ATCC 43612, IFAM G-1381.

GenBank accession number (16S rRNA): X97692.

Additional Remarks: Other strains include IFAM BA-868 (ATCC 43613) and IFAM BA-869 (ATCC 43615).

3. ***Pedomicrobium australicum*** Gebers and Beese 1988, 313^{VP} *au.stra'li.cum*. M.L. neut. adj. *australicum* isolated from Australia.

Cells oval, tetrahedral, short rods, bean- or spindle-shaped, 1.2 × 1.8 µm with up to 3 hyphae originating laterally or polarly. Cells may produce up to 3 buds at the same time; intercalary budding has been observed. Motile swimmers have one subpolar flagellum. Extracellular polyanionic polymer is produced primarily on the cell surface opposite the lateral hypha (Fig. BXII.α.221). Iron oxides are deposited on the surface when cells are grown on fulvic acid iron sesquioxide complexes or in the presence of 5 mg/l of FeSO₄·7H₂O. Deposition of manganese oxides occurs on PSM agar in the presence of 1.54 mg/l MnSO₄·H₂O. Ultrathin sections reveal intracytoplasmic membranes, which form compartments, possibly due to unfavorable growth conditions.

The temperature optimum for growth is 29–32°C; the range is 15–36°C. The optimal pH for growth in buffered media is 7.3–7.6. Utilization of carbon sources varies between the two strains (Table BXII.α.182): both grow with acetate, D,L-malate, lactose, and gluconate, but not with D-lactate, citrate, or D-ribose. Good growth occurs with (w/v)

0.05% yeast extract or with 0.025% yeast extract plus 0.025% peptone. Organic nitrogen sources include several amino acids (Table BXII.α.181); ammonia may serve as a nitrogen source, but nitrate does not. Strain WD-1355 is stimulated by cyanocobalamin (1 µg/l) and by the presence of 0.1% NaCl. Both strains are positive for catalase and for nitrate reduction. The strains are inhibited by a variety of antibiotics (Table BXII.α.184), but not by sulfanilamide.

DNA–DNA similarity studies (Table BXII.α.183) clearly demonstrate species identity between the two isolates.

Habitat: a freshwater reservoir in New South Wales, Australia.

The mol% G + C of the DNA is: 65 (ST-1306^T) and 63 (WD-1355) (T_m).

Type strain: ATCC 43611, IFAM ST-1306.

GenBank accession number (16S rRNA): X97693.

Additional Remarks: Other strains include IFAM WD-1355 (ATCC43614).

4. ***Pedomicrobium manganicum*** Aristovskaya 1961, 957^{AL} *emend.* Gebers 1981, 315.

man.ga'ni.cum. M.L. neut. adj. *manganicum* of manganese.

Cells primarily spherical, oval, or short rods, 0.4–0.9 × 0.4–1.5 µm, with 1–5 hyphae per cell. Colonial morphology as for the genus. Cells have polyanionic polymer and manganese oxide deposits, but only rarely deposits of iron oxide. Buds may be formed intercalary in hyphae. Intracytoplasmic membranes occur. Cells grow on acetate, caproate, formate, D-glucose, D-lactate, pyruvate, and D-ribose. Optimal growth temperature 30°C. Growth is stimulated by vitamin mixtures, but inhibited by gentamicin and bacitracin. Further physiological characteristics are listed in Tables BXII.α.181, BXII.α.182, BXII.α.183, BXII.α.184, BXII.α.185, and BXII.α.186.

Isolated from a quartzite rock pool in France.

The mol% G + C of the DNA is: 65 (T_m).

Type strain: ACM 3038, ATCC 33121, DSM 1545, IFAM E-1129.

GenBank accession number (16S rRNA): X97691.

Genus XV. ***Prosthecomicrobium*** Staley 1968, 1940^{AL} *emend.* Staley 1984, 304

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Pros.the'co.mi.cro.bi.um. Gr. fem. n. *prosthece* appendage; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. neut. n. *Prosthecomicrobium* appendage (-bearing) microbe.

Unicellular bacterium with coccobacillary to rod-shaped cells ranging in diameter from 0.8 to 1.2 µm and containing **numerous prosthecae** extending from all locations on the cell surface. Prosthecae, which may number from 10 to more than 30/cell, are typically short (i.e., <1.0 µm in length); some species, however, also produce longer prosthecae (>2.0 µm). **Cells divide by budding**. Buds are produced directly from the mother cell, never from tips of prosthecae. **Gram negative**. Motile and nonmotile species exist. Motile organisms produce single polar to subpolar flagella; one species forms gas vacuoles but not flagella. **Obligately aerobic, nonfermentative, heterotrophic**. A variety of sugars and organic acids are used as energy sources for growth. All strains tested require one or more B vitamins for growth. Oxidase and catalase positive. Found in soils and fresh and marine waters.

The mol% G + C of the DNA is: 64–70.

Type species: ***Prosthecomicrobium pneumaticum*** Staley 1968, 1940.

FURTHER DESCRIPTIVE INFORMATION

The genus *Prosthecomicrobium* comprises a diverse collection of multiply-appendaged prosthecate bacteria. All produce short prosthecae, i.e., prosthecae that are typically <1.0 µm in length. The shortest prosthecae appear as small bumps on the surface of the cell and cannot be readily discerned by light microscopic examination of cells. The “corn cob” organism (now named “*P. polysphaeroidum*”) was so named because of the numerous short stubby prosthecae that are regularly arranged on the surface of the cell. Somewhat longer prosthecae are conical in shape and are typical for many species. These range in length from <0.25 to >1.0 µm, depending on the species and the growth medium

(longer prosthecae may be produced when phosphate is limiting growth). Even longer prosthecae are produced by some strains. For example, *P. pneumaticum* occasionally produces prosthecae $>2.0\text{ }\mu\text{m}$ long. This, however, occurs rarely (Staley, 1968). Some strains of the marine species *P. litoralum* also produce longer appendages. One species, *P. hirschii*, produces both short appendaged as well as long-appendaged cells. The long-appendaged cells closely resemble those of *Ancalomicrobium adetum* and could be confused with them if morphology is used as the sole criterion for identification of organisms from natural samples.

Buds are produced as outgrowths at or near one pole of the cell (Vasilyeva, 1972a; Staley, 1984). These typically appear as a forked protuberance from the dividing pole. The bud enlarges and differentiates to produce a mirror image of the mother cell. As in *Ancalomicrobium*, buds appear to be synthesized *de novo* (Staley, 1973b; Staley et al., 1981).

Some species of *Prosthecomicrobium* produce flagella. Flagella are always single and may be found in a polar or subpolar position on the cell. One species, *P. pneumaticum*, does not produce flagella but produces gas vacuoles. Some species are nonmotile and do not produce flagella or gas vacuoles.

All species are obligately aerobic chemoorganotrophs that use a variety of sugars, organic acids, and sugar alcohols for growth. All species can grow on a simple, defined medium (Staley, 1981a). All species require one or more water-soluble vitamins for growth.

Prosthecomicrobium species are not known to be pathogenic to humans.

Prosthecomicrobium species are found in soils, freshwater habitats of all trophic states (Staley et al., 1980), as well as in the marine habitat (Bauld et al., 1983). They have been reported as important components of biofilms in sewage treatment processes and in pulp mill aeration lagoons (Stanley et al., 1979). Bacteria that resemble these forms have also been reported in the intes-

tinal tracts of insects and other animals (Cruden and Markovetz, 1981); however, it is uncertain whether they have been concentrated from the foodstuff or are truly indigenous to this environment.

ENRICHMENT AND ISOLATION PROCEDURES

The same procedures described for *Ancalomicrobium* isolation can be used for isolation of *Prosthecomicrobium*. The dilute peptone enrichment procedure has been used successfully for the isolation of most strains. Recognition of short-appendaged species in the enrichment culture is difficult because they are not as noticeable as the longer appendaged species. Nonetheless, even with the phase-contrast microscope, they can be detected because of their slightly irregular surface (Fig. BXII.α.225). However, it may be desirable to observe preparations from wet mounts with the transmission electron microscope to confirm their presence in the enrichments. When their numbers have reached significant proportions of the total numbers of bacteria (i.e., 5%), then attempts at isolation should be made. Dilute peptone agar plates should be streaked and incubated at room temperature for 2 weeks or more. Colonies are typically small. Wet mounts of each colony type should be prepared and examined by phase-contrast microscopy for identification. The morphology of the resulting pure culture should be confirmed by examination of whole cells with the transmission electron microscope. Additional information on isolation has been published elsewhere (Staley, 1981a).

MAINTENANCE PROCEDURES

Strains can be maintained on slants in the refrigerator for at least 1 month. MMB agar (Staley, 1981a) with glucose as a carbon source provides a favorable growth medium. Lyophilization can be used for long-term preservation.

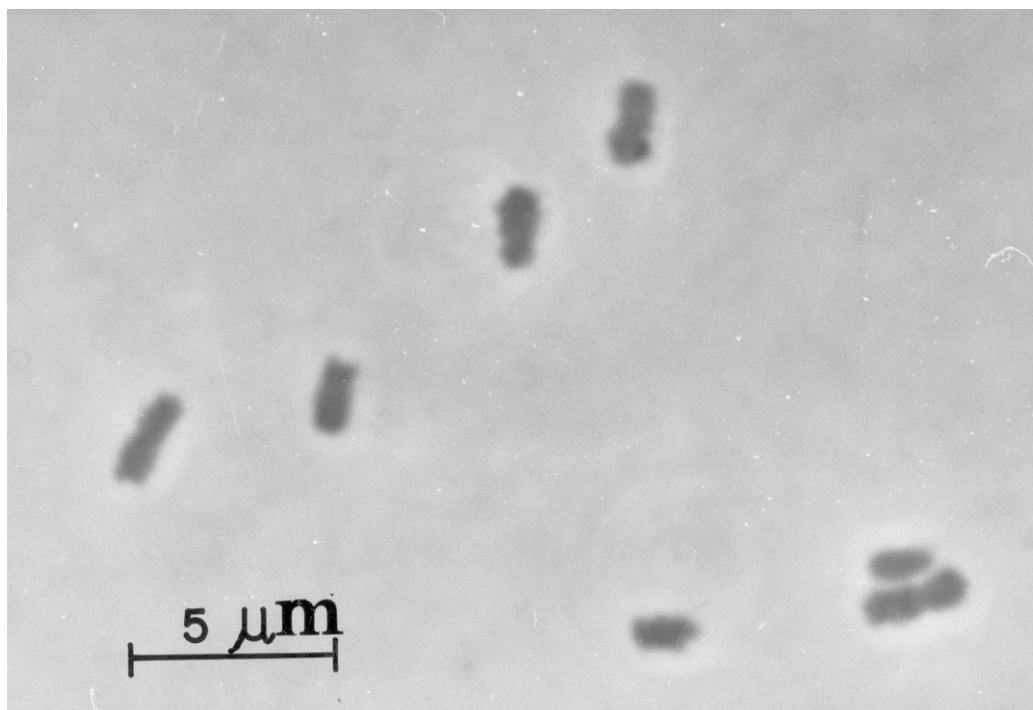


FIGURE BXII.α.225. Phase contrast photomicrograph of *P. enhydrium* showing several cells. Note the irregular surface of the cells due to the presence of numerous short prosthecae.

DIFFERENTIATION OF THE GENUS *PROSTHECOMICROBIUM* FROM OTHER GENERA

Only two genera pose major problems for differentiation. These are the genera *Ancalomicrobium* and *Verrucomicrobium*. Differentiation from the former genus is discussed in detail in the section on *Ancalomicrobium*. Morphologically, *Ancalomicrobium* can be distinguished from *Prosthecomicrobium* because of its conical cells and lack of short-appendaged cells. Furthermore, *Ancalomicrobium* is a genus of facultative anaerobes that ferments selected sugars. Thus, the Hugh-Leifson test can be used to distinguish between these two groups (cf. Table BXII.α.165 in *Ancalomicrobium*). The major phenotypic feature differentiating cells of the genus *Verrucomicrobium* from those of *Prosthecomicrobium* is the presence of fimbriae, which extend from the tips of the prosthecae. These are absent in cells of the genus *Prosthecomicrobium*.

TAXONOMIC COMMENTS

Morphologically, *Prosthecomicrobium* comprises a diverse collection of bacteria. There are differences in appendage length, motility, gas vacuole formation, habitat, and carbon source utilization among the species so far described.

The mol% G + C of the DNA within the genus ranges from 64 to 70 (Staley and Mandel, 1973). Results of DNA-DNA hybridization studies (Moore and Staley, 1976) suggest that additional species exist that have not yet been fully described. Several unnamed strains have been deposited in the ATCC (ATCC 27825, 27826, and 27833). *Ancalomicrobium adetum* shows only low levels of homology with *Prosthecomicrobium* species that have been tested.

Phylogenetic analysis of the 16S ribosomal RNA gene sequences of *P. pneumaticum*, *P. enhydrium*, and several uncharac-

terized *Prosthecomicrobium*-like strains demonstrates that these organisms belong to the *Alphaproteobacteria*. Morphologically similar organisms of the genus *Ancalomicrobium* also belong to the *Alphaproteobacteria* but form a separate and distinct genus based on 16S rRNA analyses. These findings are consistent with earlier 16S rRNA cataloging studies on these genera (Schlesner et al., 1989). Members of the genus *Verrucomicrobium* are not phylogenetically affiliated with *Prosthecomicrobium* strains, despite being morphologically similar, and fall within a separate bacterial division.

Analyses of the 16S rRNA genes of *Prosthecomicrobium* strains also demonstrate that the genus *Prosthecomicrobium* is polyphyletic (Fig. BXII.α.226). Of the strains that have been sequenced, only strain P4.10, an isolate from a pulp mill oxidation lagoon, clusters with the type species, *P. pneumaticum*. Four additional strains, including *P. enhydrium*, cluster with *Devosia riboflavina* (formerly "*Pseudomonas riboflavina*"), a non-prosthecae organism. One additional strain SCH71 does not cluster with either of these groups. Thus, the taxonomy of members of the genus *Prosthecomicrobium* should be reexamined.

16S rRNA sequencing of the remaining characterized species *P. litorale* and *P. hirschii* has not yet been completed and is required to assess the extent of diversity within the *Prosthecomicrobium* genus. Such studies would also help to clarify the taxonomic relationships of the strains "*P. polysphaeroidum*", "*P. consociatum*", "*P. mishustinii*", which are at present only unofficially named (Vasilyeva and Lafitskaya, 1976; Vasilyeva et al., 1991).

Reclassification of *P. enhydrium* and related strains is also required as they do not cluster with the type species of *Prosthecomicrobium*, *P. pneumaticum*. These strains should therefore be reassigned to a different genus.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *PROSTHECOMICROBIUM*

The characteristics differentiating among the species of the genus *Prosthecomicrobium* are listed in Table BXII.α.187.

List of species of the genus *Prosthecomicrobium*

1. ***Prosthecomicrobium pneumaticum*** Staley 1968, 1940^{AL}
pneu.ma'ti.cum. N.L. adj. *pneumaticum* inflated, containing gas vacuoles.

Most prosthecae are short (i.e., <1.0 μm in length); however, long prosthecae are occasionally produced (Fig. BXII.α.227). Gas vacuoles are formed by the type strain. Flagella are not produced. Ammonium, but not nitrate, can be used as a sole nitrogen source for growth. A variety of sugars, both hexoses and pentoses, monosaccharides and disaccharides, can be used as carbon sources for growth (Table BXII.α.187). Sugar alcohols and methyl sugars fucose and rhamnose can also be used. Biotin, thiamine, and vitamin B₁₂ are required for growth. The temperature range for the type strain is 9–42°C. Optimal pH: 6.0–6.5, although good growth also occurs at pH 7.0. Colonies are translucent to opaque white (the chalky white color is due to the formation of gas vacuoles). Colony size is quite variable.

The mol% G + C of the DNA is: 69–70 (Bd).

Type strain: ATCC 23633, VKM B-1389.

2. ***Prosthecomicrobium enhydrium*** Staley 1968, 1940^{AL}
en.hy'drum. N.L. adj. *enhydrium* living in water, aquatic.

Prosthecae are always short, i.e., <0.5 μm, giving the cell an irregular surface when observed by phase-contrast mi-

croscopy (Fig. BXII.α.225). Cells are motile by a polar to subpolar flagellum (Fig. BXII.α.228). Gas vacuoles are not produced. Ammonium but not nitrate can be used as a sole source of nitrogen in media containing an appropriate carbon source and vitamins. Pentoses and hexoses are commonly used by the type strain. Some disaccharides and organic acids are also used as carbon sources (Table BXII.α.187). Thiamine is required for growth. The temperature range for growth of the type strain is 9–37°C. The pH optimum for growth is 7.0. Colonies may be white (type strain) or pigmented yellow or red.

The mol% G + C of the DNA is: 65.8 (Bd).

Type strain: ATCC 23634, VKM B-1376.

3. ***Prosthecomicrobium hirschii*** Staley 1984, 304^{VP}
hirsch'i.i. N.L. gen. *hirschii* of Hirsch; named in honor of P. Hirsch, an authority on budding bacteria.

Prosthecae may be short (i.e., < 1.0 μm in length) or long (>2.0 μm in length), depending on the cell. Both short- and long-appendaged cells occur simultaneously in culture (Fig. BXII.α.229). Cells may be motile by single polar or subpolar flagella. Gas vacuoles are not formed. Sugars and organic acids are commonly used as carbon sources. Long chain organic acids such as valerate and caproate are used. Methanol and ethanol can also be used as

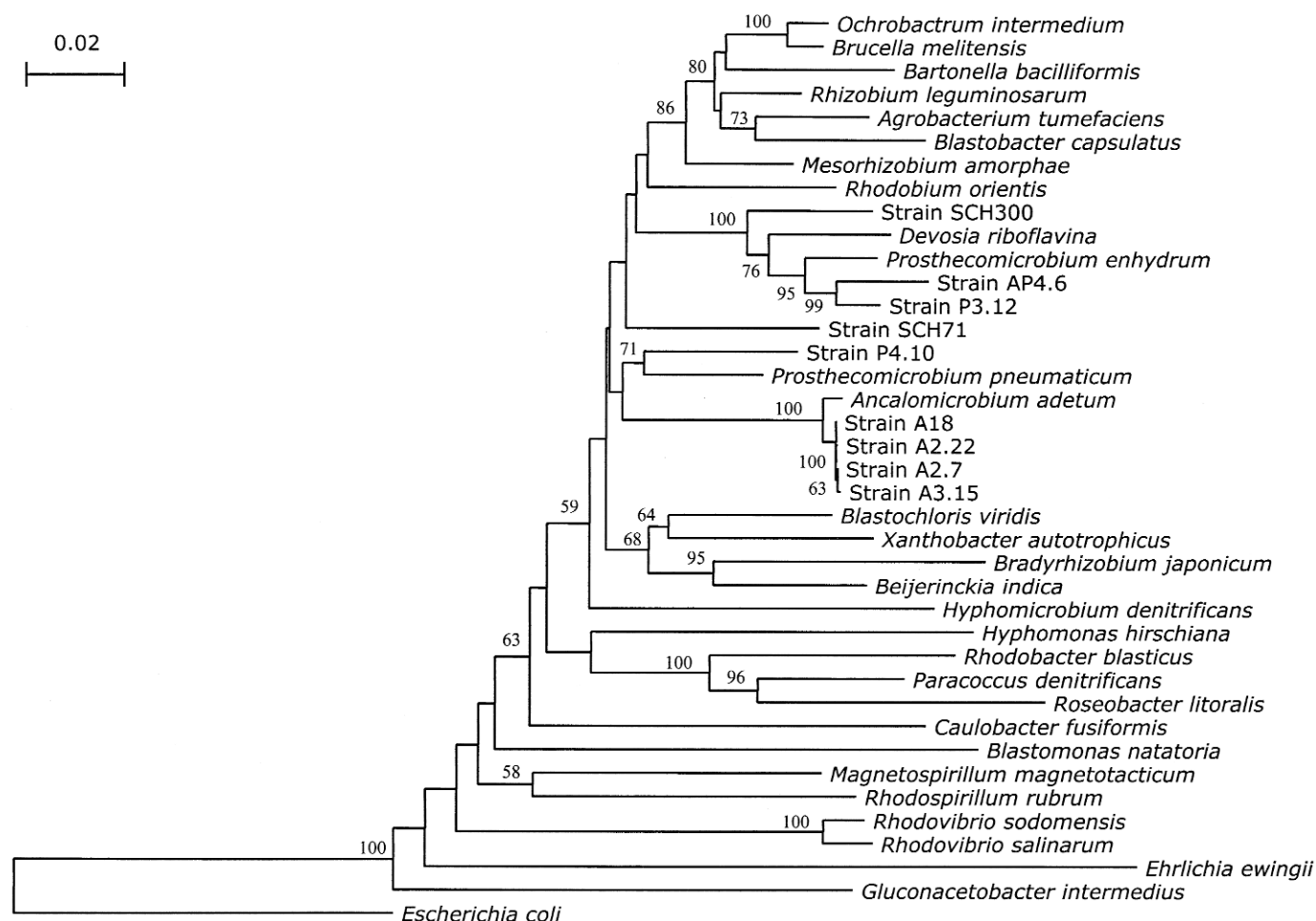


FIGURE BXII.α.226. Phylogenetic tree of 16S rRNA sequences showing the positions of various *Prosthecomicrobium* strains within the *Alphaproteobacteria*. The genus is polyphyletic, with the type strain *P. pneumaticum* and the pulp mill aeration lagoon isolate P4.10 forming one clade, while *P. enhydrum* and the *Prosthecomicrobium*-like strains AP4.6, P3.12, and SCH300 form a separate clade that includes the nonprosthecate organism *Devosia riboflavina*. Strain SCH71, which is also *Prosthecomicrobium*-like in morphology, forms a third group with no clear phylogenetic affiliation. The tree was constructed using Kimura-2-parameter distances and the neighbor joining algorithm with 100 bootstrap replications within the program TreeCon. Bootstrap values above 50% are shown. Bar indicates 0.02 substitutions per site.

TABLE BXII.α.187. Characteristics differentiating species of the genus *Prosthecomicrobium*^a

Characteristic	<i>P. enhydrum</i>	<i>P. hirschii</i>	<i>P. litoralis</i>	<i>P. pneumaticum</i>	<i>"P. polyspheroidum"</i>	<i>"P. consociatum"</i>	<i>"P. mishustinii"</i>
Short prosthecae (<1 μm) on MMB	+	+	+	+	+	+	+
Long prosthecae (>2 μm) on MMB	—	—	— ^b	—	—	—	—
Lateral buds	—	—	—	—	+	—	—
Flagella	+	+	—	—	+	—	—
Gas vacuoles	—	—	—	+	—	—	—
Sodium ion requirement; optimal salinity of 25‰	—	—	+	—	—	—	—
<i>Carbon source utilization:</i>							
Maltose, cellobiose, lactose	+	+	+	+	+	—	+
Melibiose	—	—	+	+	nd	—	—
Rhamnose	+	—	+	+	—	±	±
Sorbitol	—	—	+	+	±	—	—
Pyruvate	+	+	+	—	—	—	±
Propionate	—	+	—	—	±	—	—
Agar digestion	—	nd	+	—	—	—	—
Mol% G + C of DNA	66	68–70	66–67	69–70	64–67	66–68	64–65

^aSymbols: +, 90% or more strains are positive; —, 90% or more strains are negative; nd, not determined; ±, indefinite.

^bStrains of both species rarely produce long appendages.

^cThese are formed rarely.

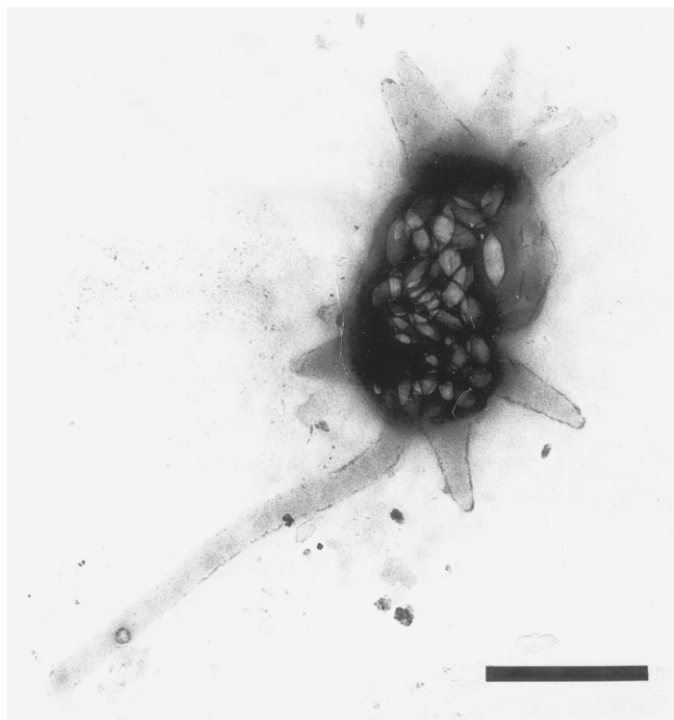


FIGURE BXII.α.227. *P. pneumaticum* as observed by electron microscopy. Note the gas vesicles within the cell and the single long prostheca. Bar = 1.0 μ m.

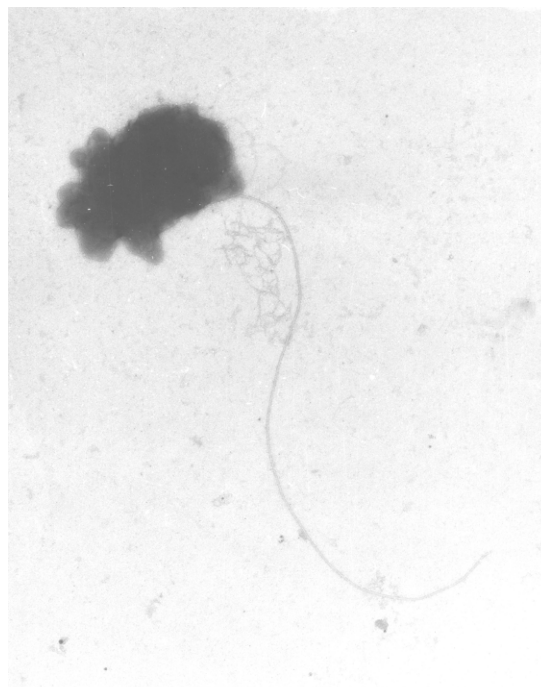


FIGURE BXII.α.228. Electron micrograph of *P. enhydrium*. Note the sub-polar location of the single flagellum.

carbon sources. Biotin, nicotinic acid, pantothenate, and thiamine are required for growth. Colonies are circular in form and umbonate in elevation. They have an entire margin and are pink in color.

The mol% G + C of the DNA is: 67.9–69.9 (Bd).

Type strain: ATCC 27832.

4. *Prosthecomicrobium litoralum* Bauld, Bigford and Staley 1983, 613^{VP}

li.to.ra' lum. L. adj. *litoralis* of the seashore.

Prosthecae are mostly short (i.e., <0.5 μ m in length), although occasionally long prosthecae are formed. Non-motile. Neither flagella nor gas vacuoles are produced. Ammonium can be used as a sole source of nitrogen. A variety of pentoses and hexoses can be used for growth as well as some sugar alcohols and organic acids (Table BXII.α.187). Agar digestion occurs on prolonged incubation. Sodium ions are required for growth. The minimum salinity at which growth occurs is 5‰ the optimum, 25‰ (Table BXII.α.187). These bacteria are psychrotrophic. Their minimum temperature for growth is 1–5°C, depending on the strain, and their optimum is between 27° and 34°C. Colonies are white and raised initially and, after prolonged incubation, become cream-colored and umbonate. Agar digestion is noticeable around colonies on plates incubated at least 30 days at room temperature.

The mol% G + C of the DNA is: 66–67 (Bd).

Type strain: 524-16, ATCC 35022.

5. “*Prosthecomicrobium polyspheroidum*” (Nikitin and Vasilyeva 1968) Vasilyeva and Lafitskaya 1976, 768 (“**Agrobac-**

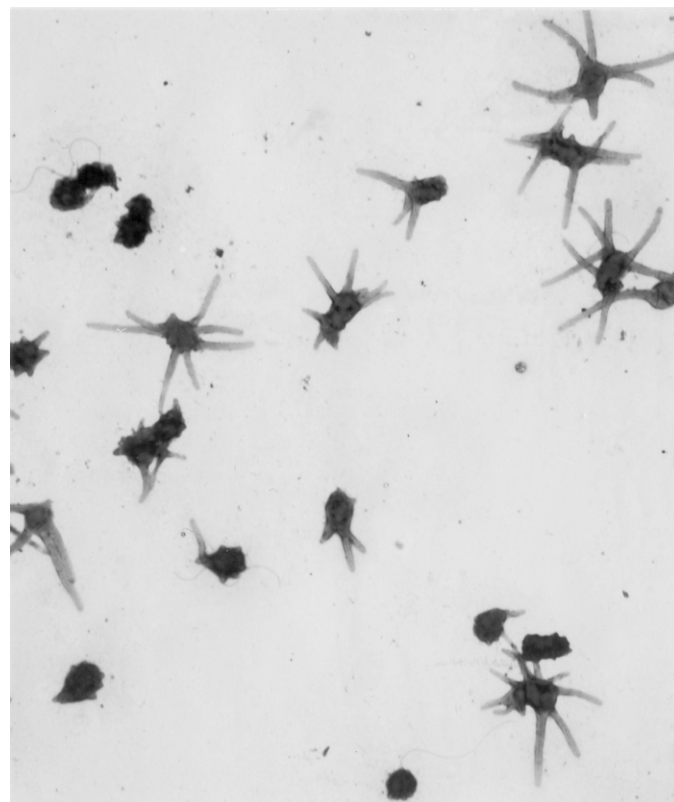


FIGURE BXII.α.229. Electron micrograph of *P. hirschii* showing short-appendaged cells, long-appendaged cells, and flagella.

terium polyspheroidum” Nikitin and Vasilyeva 1968, 444.) *po.ly.spher.oi'dum*. N.L. adj. *polyspheroidum* many spheroids, i.e., having numerous bumps.

Cells have many (up to 230/cell) short prosthecae (i.e., $<0.1\ \mu\text{m}$ in length). They are distributed in slightly spiral rows along the length of the rod-shaped cells, giving the appearance of a corn cob (Fig. BXII.α.230). Cells, which measure about $0.5\ \mu\text{m}$ in diameter and up to $5.1\ \mu\text{m}$ in length, are motile by a polar flagellum. Lateral buds may be formed under some conditions of growth. Prosthecae may not be evident in cultures containing more than 0.1% organic nutrients. Monosaccharides and disaccharides are metabolized by pentose phosphate and the Entner–Doudoroff pathway, with the formation of alcohols, organic acids, and aromatics (cf. Lafitskaya et al., 1976). Thiamine and riboflavin are required for growth. Nitrate reduced to nitrite. Voges–Proskauer and methyl red tests are negative. Colonies are punctiform after 2–3 days of growth and attain a diameter of about 1 mm after 10 days. They are raised with entire margins, opaque, cream colored, and slimy. Optimal temperature: $28\text{--}30^\circ\text{C}$; optimal pH: 6.5–7.1.

The mol% G + C of the DNA is: 64 to 67 (Bd).

Deposited strain: AUCM B-1313.



FIGURE BXII.α.230. Electron micrograph of “*P. polysphaeroidum*”. Note the arrangement of the numerous short prosthecae in rows. Bar = $2.0\ \mu\text{m}$. (Photograph courtesy of L.V. Vasilyeva.)

Genus XVI. Rhodomicrobium Duchow and Douglas 1949, 415^{AL} emend. Imhoff, Trüper and Pfennig 1984, 341

JOHANNES F. IMHOFF

Rho.do.mi.cro'bi.um. Gr. n. *rhodon* the rose; Gr. adj. *micros* small; Gr. n. *bios* life; M.L. neut. n. *Rhodomicroibum* red microbe.

Ovoid to elongate-ovoid bacteria showing polar growth and performing a characteristic vegetative growth cycle. This cycle includes the formation of peritrichously flagellated swarmer cells and nonmotile “mother cells”, which form prosthecae from one to several times the length of the mother cell. Daughter cells originate as spherical buds at the end of the prosthecae and may undergo differentiation in various ways. **Gram negative, belonging to the Alphaproteobacteria.** Internal photosynthetic membranes are of the lamellar type. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. The predominant cellular fatty acid is $\text{C}_{18:1}$, which comprises more than 80% of the membrane-bound fatty acids. Ubiquinone and rhodoquinone with 10 isoprene units are present, and the lipopolysaccharides are characterized by a glucosamine-containing, phosphate-free lipid A with amide-bound $\text{C}_{16:0}\ 3\text{OH}$.

Cells grow preferably photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth may be possible with hydrogen and sulfide as electron sources. Various organic substrates, molecular hydrogen, ferrous iron, and sulfide at low concentrations may be used as photosynthetic electron donors. Cells are also able to grow under microoxic to oxic conditions in the dark.

Mesophilic freshwater bacteria with a preference for acidic pH, between 5.2 and 6.5.

The mol% G + C of the DNA is: 61.8–63.8.

Type species: ***Rhodomicrobium vannielii*** Duchow and Douglas 1949, 415.

FURTHER DESCRIPTIVE INFORMATION

Rhodomicrobium vannielii is the sole species of this genus. Its morphology and growth cycle resemble those of the nonphototrophic *Hyphomicrobium* species (Hirsch, 1974a). The characteristic growth cycle has been the object of intensive studies (Whittenbury and Dow, 1977) and is outlined in the species description. *Rhodomicrobium vannielii* grows best under anoxic conditions in the light with a number of carbon sources at low light intensities and acidic pH (5.5–6.5). Dinitrogen and ammonia are the best nitrogen sources. Ammonia is assimilated via glutamine synthetase/glutamate synthase (NADH-dependent); low activities of a glutamate dehydrogenase (NADPH-dependent) are also present (Brown and Herbert, 1977). Sulfate can be used as the sole sulfur source and is assimilated via adenosine-5'-phosphosulfate (Imhoff, 1982). Sulfide is tolerated at 2–3 mM and oxidized exclusively to tetrathionate in sulfide-limited chemostat culture. In batch culture, sulfide may react with the tetrathionate formed, and under these conditions, thiosulfate and elemental sulfur are the major end products (Hansen, 1974).

Particular similarities have been found in the amino acid sequences of the “small type” cytochromes c_2 of *Rhodomicrobium vannielii*, *Blastochloris viridis*, and *Rhodoblastus acidophilus* (Ambler et al., 1979). As in a few other purple nonsulfur bacteria (*Rhodospira globiformis*, *Rubrivivax gelatinosus*, and *Rhodocyclops tenuis*), a high potential iron–sulfur protein (HiPIP) is present (T.E. Meyer, personal communication). High proportions of unusual aminolipids have been found in *Rhodomicrobium vannielii* (Park and

Berger, 1967; Imhoff et al., 1982). The fatty acid composition is characterized by an extremely high proportion of C_{18:1}, comprising more than 85% of the membrane-bound fatty acids, as in *Hyphomicrobium* species, *Pedomicrobium* species, "*Nitrobacter agilis*", and *Nitrobacter winogradskyi* (Auran and Schmidt, 1972; Eckhardt et al., 1979). Ubiquinone and rhodoquinone with 10 isoprene units are present (Hiraishi and Hoshino, 1984). As is the case with *Rhodoblastus acidophilus*, but not with most of the phototrophic *Alphaproteobacteria*, the lipid A of *R. vannielii* contains glucosamine (not 2,3-diamino-2,3-dideoxyglucose) as the sole amino sugar and amide-bound C_{16:0 3OH} (Weckesser et al., 1995).

A new isolate, which has been tentatively identified as *R. vannielii* and grown in the light with ferrous iron as the sole energy source, has been found to use ferrous iron as a photosynthetic electron donor, as has the type strain (Widdel et al., 1993; Heising and Schink, 1998). It has been noted that growth is not supported over prolonged time periods when ferrous iron is the exclusive electron source, and that acetate and succinate stimulate growth with ferrous iron (Heising and Schink, 1998). Ferric iron is not reduced in the dark, and manganese salts are neither oxidized nor reduced.

ENRICHMENT AND ISOLATION PROCEDURES

Rhodomicrobium is commonly found in mud and water of ponds and lakes, as well as in wastewater. About 50% of enrichment cultures with freshwater sediments prove to contain *Rhodomicrobium* species (Whittenbury and Dow, 1977). *Rhodomicrobium* has also been isolated from brackish-water and seawater habitats (Hirsch and Rheinheimer, 1968).

Enrichment, isolation, and growth occur under conditions suitable for most of the other species of the purple nonsulfur bacteria (see chapter Genus *Rhodospirillum*) with organic acids or other organic substrates as carbon and electron sources (Imhoff, 1988; Imhoff and Trüper, 1992). For selective enrichments, a succinate–mineral medium with an initial pH of 5.2–5.5 should be used, and growth factors should be omitted (Pfennig, 1969a).

MAINTENANCE PROCEDURES

Cultures of *Rhodomicrobium vannielii* are well preserved in liquid nitrogen, by lyophilization, or at -80°C in a mechanical freezer. Late-exponential-phase cell suspensions should be mixed with glycerol or DMSO to yield the final cryoprotectant concentrations of 10% and 5%, respectively, kept at 0°C for 15 min, and then frozen immediately.

DIFFERENTIATION OF THE GENUS *RHODOMICROBIUM* FROM OTHER GENERA

The lamellar structure of the internal photosynthetic membranes, the polar growth mode, and the budding type of multiplication are similar to those of other budding purple nonsulfur bacteria. Prostheca formation and the characteristic growth cycle are the most obvious distinguishing properties. In addition, according to rRNA–DNA hybridization studies, *Rhodomicrobium* is clearly distinguished from other purple nonsulfur bacteria (Gillis et al., 1982).

Among the closest phylogenetic relatives of *Rhodomicrobium*, based on 16S rDNA sequence analysis, are *Hyphomicrobium vulgare*, *Blastochloris viridis*, and *Rhodopseudomonas palustris* (Kawasaki et al., 1993b). Other outstanding characteristics are the compositions of lipid A, polar lipids, and fatty acids. Major differentiating properties between *Rhodomicrobium* and other phototrophic *Alphaproteobacteria* are shown in Tables BXII.α.168 and BXII.α.169 of the chapter on the genus *Blastochloris* as well as in Tables 3 (pp. 125–126) and 4 (p. 127) in the introductory chapter "Anoxygenic Phototrophic Purple Bacteria", Volume 2, Part A. The phylogenetic relationships of these bacteria, based on 16S rDNA sequences, are shown in Fig. 2 (p. 128) of that chapter.

FURTHER READING

- Gorlenko, V.M., N.N. Egorova and A.N. Puchkov. 1974. Fine structure of exospores of nonsulfur purple bacterium *Rhodomicrobium vannielii*. *Mikrobiologia*. 43: 913–915.
- Hirsch, P. 1974. Budding bacteria. *Ann. Rev. Microbiol.* 28: 391–444.
- Whittenbury, R. and C.S. Dow. 1977. Morphogenesis and differentiation in *Rhodomicrobium vannielii* and other budding and prosthecate bacteria. *Bacteriol. Rev.* 41: 754–808.

List of species of the genus *Rhodomicrobium*

1. ***Rhodomicrobium vannielii*** Duchow and Douglas 1949, 415^{AL}.
van.niel' i.i. M.L. gen. n. *vannielii* of van Niel; named for C.B. van Niel, an American microbiologist.

Mature cells are ovoid to lemon-shaped, $1.0\text{--}1.2 \times 2.0\text{--}2.8\text{ }\mu\text{m}$, and multiply by polar growth and budding (Fig. BXII.α.231). Cells perform a characteristic vegetative growth cycle; the motile, peritrichously flagellated swarmer cells, which lose their flagella later in the growth cycle, form prosthecae of about $0.3\text{ }\mu\text{m}$ in diameter, and a daughter cell arises as a spherical bud at the end of each prostheca. This daughter cell may separate from the prostheca and start a new cycle as a swarmer cell. After the swarmer cell is released, the pole of the prostheca is free for the formation of another bud. Alternatively, the daughter cell may remain attached to the prostheca and form another prostheca at the opposite pole. Branching of prosthecae may occur by lateral outgrowth of new prosthecae from the primary prostheca, upon which the first daughter cell formed. A branched prostheca can be formed only on the most

recently synthesized prostheca, and plug formation occurs in the prostheca of each daughter cell before that prostheca branches to form the next daughter cell. Only one daughter cell is formed at a time. Because cells tend to remain attached to the prosthecae, aggregates containing large numbers of cells are usually formed. In addition, smaller cells, called exospores, may eventually be produced. These polyhedral cells are $1.0\text{--}1.5\text{ }\mu\text{m}$ in diameter. One to four such cells are formed sequentially as buds at a common branching point at the end of a prostheca (Fig. BXII.α.231). The exospores are more resistant to dryness and heat than are normal vegetative cells.

Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane. No lamellae are present in the prosthecae. The color of cell suspensions is from salmon pink to deep orange-brown to red. Absorption spectra of living cells have maxima at 378, 461, 488–490, 522–525, 800–807, and 869–872 nm. Aerobically grown cells are colorless to pale orange-brown. Photosynthetic pigments are bacteriochlorophyll *a* (esteri-

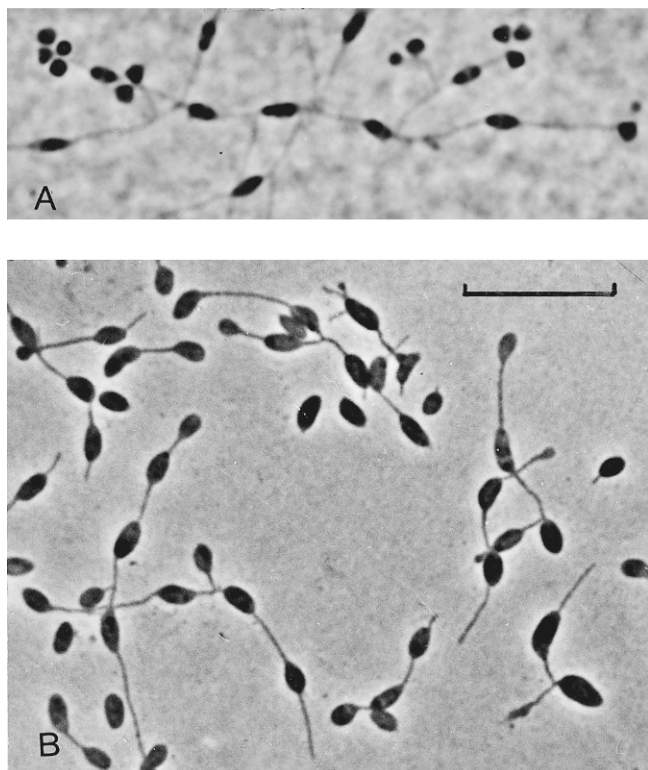


FIGURE BXII.α.231. *Rhodomicrobium vannielii* strain DSM 163. A, polyhedral exospores as buds at the ends of short filaments with common branching points. B, cells with filaments and buds of various sizes. Phase-contrast micrographs. Bar = 10 μ m. (Courtesy of N. Pfennig.)

fied with phytol) and carotenoids of the spirilloxanthin series, with rhodopin as the major component and with small amounts of beta-carotene.

Cells grow preferably photoheterotrophically under anoxic conditions in the light, but photoautotrophic growth and chemotrophic growth under microoxic to oxic conditions is possible. Substrates used are acetate, propionate, butyrate, valerate, caproate, caprylate, ethanol, propanol, butanol, lactate, pyruvate, malate, fumarate, succinate, and malonate. Some strains also use methanol, formate, oxalacetate, OH-butyrate, and glycerol. Not used are citrate, tartrate, fructose, glucose, mannose, mannitol, sorbitol, glycolate, oxalate, pelargonate, benzoate, aspartate, arginine, and glutamate. Addition of HCO_3^- is essential only when CO_2 cannot be generated from the organic carbon source.

Molecular hydrogen and sulfide may serve as electron donors for photoautotrophic growth. The latter is oxidized to tetrathionate in sulfide-limited continuous culture, but thiosulfate and elemental sulfur are formed as major oxidation products in batch culture. In addition, ferrous iron is oxidized in the light, but may not support growth over prolonged times if supplied as the exclusive electron donor. Growth with ferrous iron is stimulated by acetate and succinate. Ammonia and dinitrogen, as well as Casamino acids and yeast extract, are used as nitrogen sources. Some strains grow poorly with nitrate and urea. Sulfate can serve as the sole sulfur source. No organic growth factors are required.

Mesophilic freshwater bacteria with a preference for slightly acidic pH showing optimal growth at 30°C and pH 6.0 (pH range: 5.2–7.5).

The mol% G + C of the DNA is: 61.8–63.8 (Bd).

Type strain: ATCC 17100, DSM 162.

Genus XVII. *Rhodoplanes* Hiraishi and Ueda 1994b, 671^{VP}

AKIRA HIRAISHI AND JOHANNES F. IMHOFF

Rho.do.pla' nes. Gr. n. *rhodon* rose; Gr. n. *planos* a wanderer; M.L. masc. n. *Rhodoplanes* a red wanderer.

Cells are rod-shaped, motile by means of polar, subpolar, or lateral flagella, and multiply by budding and asymmetric cell division. They are Gram-negative and belong to the *Alphaproteobacteria*. Internal photosynthetic membranes are present as lamellar stacks parallel to the cytoplasmic membrane (Fig. BXII.α.232). Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Straight-chain, monounsaturated $\text{C}_{18:1}$ is the predominant component of the cellular fatty acids and $\text{C}_{16:0}$ is a second major component. Ubiquinones and rhodoquinones with 10 isoprene units (Q-10 and RQ-10) are present.

Growth is preferably photoheterotrophic under anoxic conditions in the light, with simple organic substrates as carbon and electron sources. Photoautotrophic growth with sulfide as the electron donor does not occur. Chemotrophic growth is possible under oxic conditions in the dark at full atmospheric oxygen tension and by denitrification under anoxic conditions in the presence of nitrate. Growth factors may be required.

Mesophilic freshwater bacteria with preference for neutral pH. Some representatives may be thermotolerant.

Habitats: freshwater and wastewater environments.

The mol% G + C of the DNA is: 66.8–69.7.

Type species: *Rhodoplanes roseus* (Janssen and Harfoot 1991) Hiraishi and Ueda 1994b, 671 (*Rhodopseudomonas rosea* Janssen and Harfoot 1991, 27.)

FURTHER DESCRIPTIVE INFORMATION

Based on the 16S rDNA sequence information, *Rhodoplanes* species belong to the *Alphaproteobacteria* (Hiraishi and Ueda, 1994b). The nearest phylogenetic neighbors are *Blastochloris* species, which show approximately 94% identity of 16S rDNA sequences to those of *Rhodoplanes* species.

Although all *Rhodoplanes* species reproduce by budding and asymmetric cell division, the mode of budding differs somewhat with the species (Fig. BXII.α.233). *Rhodoplanes roseus* exhibits a sessile budding mode without formation of prosthecae, like *Rhodobium marinum* and *Rhodobacter blasticus*. *Rhodoplanes elegans*, however, does form prosthecae and rosette-like cell aggregates similar to those of *Blastochloris* species and *Rhodopseudomonas palustris*.

Cells of *Rhodoplanes* species are motile in young cultures, but motile cells become extremely rare at the late-exponential phase of growth. Electron microscopy of negatively stained cells shows that cells with single polar or subpolar flagella are dominant (Fig. BXII.α.234). In addition, cells having two lateral or subpolar

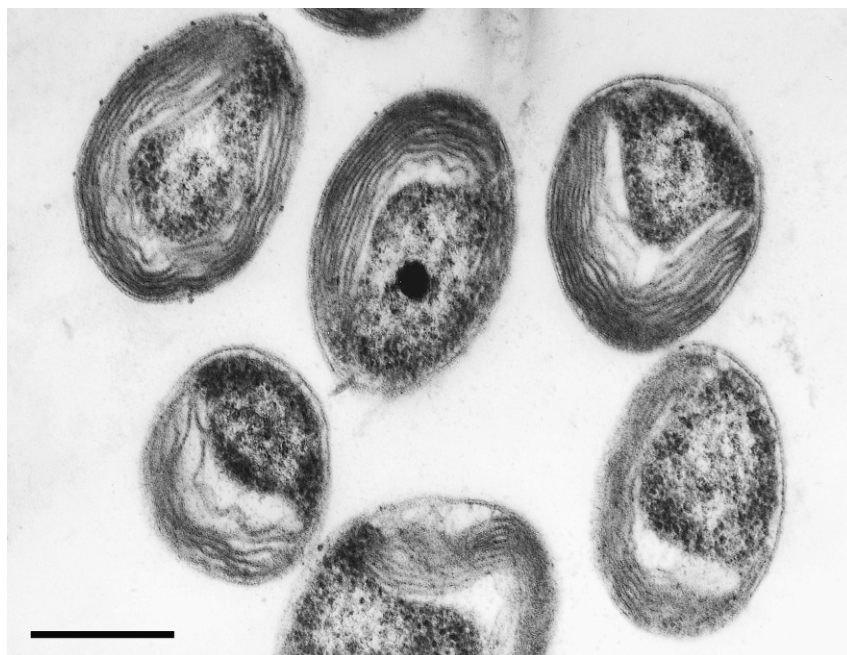


FIGURE BXII.α.232. Thin-section electron micrograph showing the internal photosynthetic membrane system of *Rhodoplanes roseus* DSM 5909. The lamellar type of photosynthetic membranes are observed. Bar = 0.5 μm .

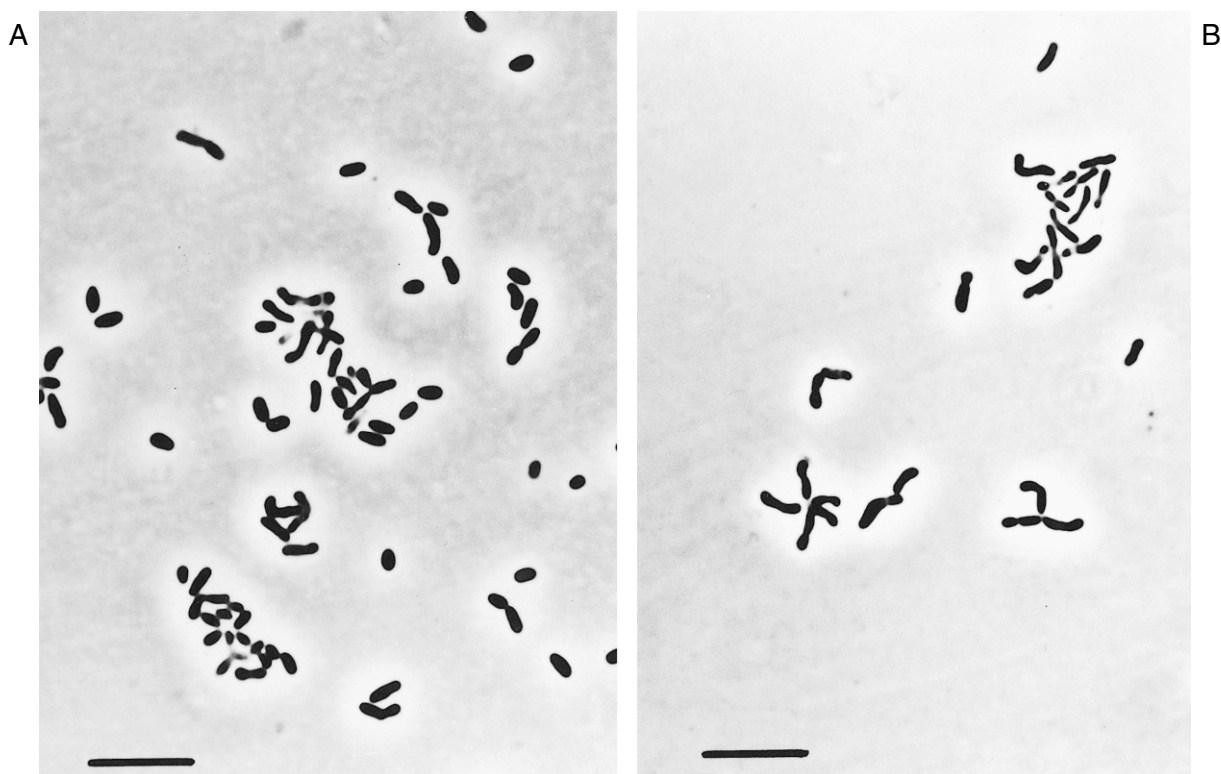


FIGURE BXII.α.233. Phase-contrast photomicrographs showing cell morphology of *Rhodoplanes*. A, *Rhodoplanes roseus* DSM 5909; B, *Rhodoplanes elegans* AS130. Bars = 8 μm .

flagella are frequently observed in *R. elegans* (Fig. BXII.α.235). Randomly distributed flagella are rarely found.

Photoheterotrophy, with simple organic compounds as electron donors and carbon sources, is the preferred mode of growth.

Pyruvate is the best substrate. Other simple organic acids, including acetate, lactate, and intermediates of the tricarboxylic acid cycle serve as good carbon sources. However, no growth occurs on sugars or sugar alcohols. While other species of pho-

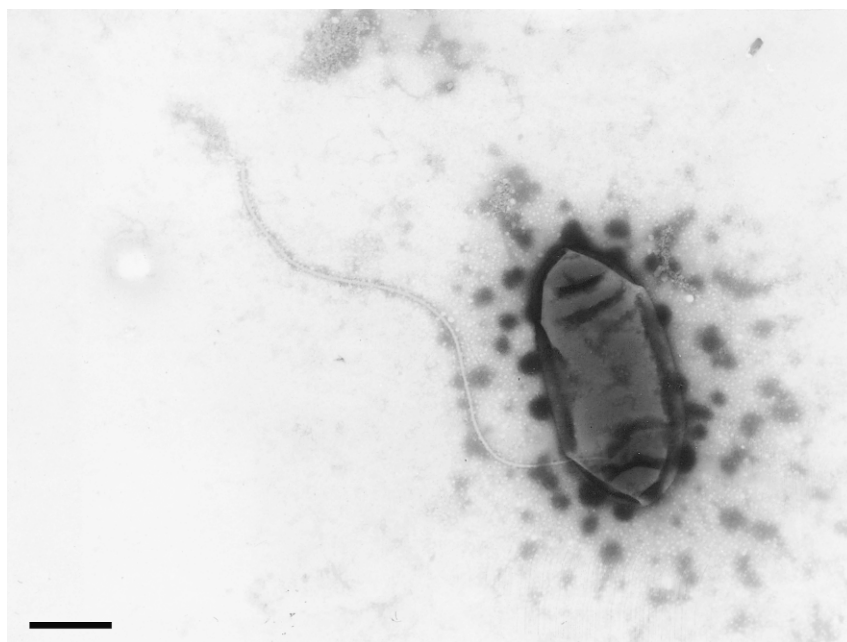


FIGURE BXII.α.234. Electron micrograph of a negatively stained cell of *Rhodoplanes roseus* DSM 5909 having a polar flagellum. Bar = 1 μm .

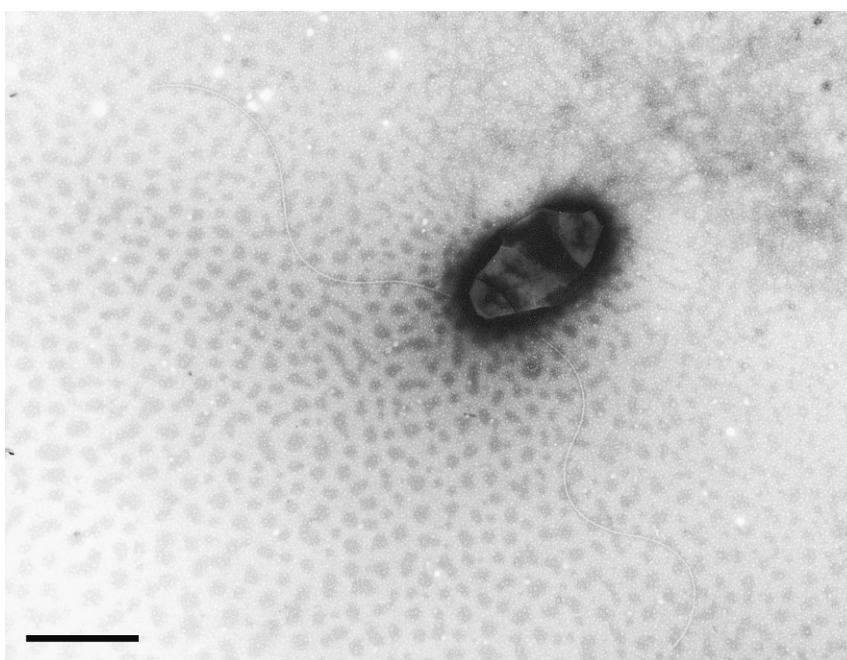


FIGURE BXII.α.235. Electron micrograph of a negatively stained cell of *Rhodoplanes elegans* AS130 having two subpolar flagella. Bar = 1 μm .

totrophic purple nonsulfur bacteria utilize straight-chain, saturated fatty acids as carbon sources, those acids ($>C_5$) have inhibitory effects on growth of *Rhodoplanes* (Janssen and Harfoot, 1987; 1991; Hiraishi and Ueda, 1994b).

Denitrification is a characteristic property of *Rhodoplanes* species. Growth under denitrifying conditions is very slow (doubling time >20 h) and is accompanied by little production of nitrogen gas. The growth yield under denitrifying conditions is 10–50% of the yield under the optimal phototrophic growth conditions.

Activities of denitrifying enzymes in *Rhodoplanes elegans* are much lower than those of other denitrifying phototrophic bacteria, such as *Rhodobacter sphaeroides* IL106 and *Rhodobacter azotoformans* (Hiraishi et al., 1995a).

The capacity for nitrogen fixation has not yet been studied intensively. However, this ability appears to be absent, as suggested by the observation that nitrogenase-dependent H_2 gas production does not occur in an ammonium-deficient medium (Hiraishi and Ueda, 1994b). Hydrolytic activities against starch,

gelatin, casein, and Tween 80 are absent. In *R. elegans*, cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine are present as the major phospholipids (A. Hiraishi, unpublished data). In all species, straight-chain, mono-unsaturated C_{18:1} is the predominant component of cellular fatty acids, constituting 60–70% of the total content. Considerable amounts of C_{16:0} (10–20%) are also present.

Since only a few strains of *Rhodoplanes* have hitherto been isolated and studied, their natural habitats and ecological significance are not yet fully understood. The type strain of *R. roseus* was isolated after enrichment in a Winogradsky column packed with mud from a duck pond in New Zealand (Janssen and Harfoot, 1991), whereas strains of *R. elegans* have been isolated from activated sludge (Hiraishi and Ueda, 1994b). As suggested by their sources and physiological characteristics, the natural habitats of *Rhodoplanes* species are freshwater environments highly polluted with organic matter, although sulfide-rich water bodies may not provide favorable conditions for their growth. Strains of *Rhodoplanes*, as well as those of *Rhodopseudomonas palustris*, *Rhodobacter blasticus*, and *Rhodobacter sphaeroides*, are frequently detected on agar plates inoculated with relatively high dilutions (10⁻⁵ to 10⁻⁶) of activated sludge samples (A. Hiraishi, unpublished data). This suggests that *Rhodoplanes* may play an active role in the purification process of polluted water.

Phylogenetic and chemotaxonomic analyses of phototrophic purple nonsulfur bacteria that have been isolated recently from hot springs of New Zealand and are able to grow at elevated temperatures up to 46°C (P. Charlton, personal communication) have demonstrated that they belong to the genus *Rhodoplanes*.

ENRICHMENT AND ISOLATION PROCEDURES

Rhodoplanes species can be cultivated in a chemically defined medium containing simple organic compounds, such as carbon sources and yeast extract, as growth factor supplements, as is commonly employed for other members of the phototrophic purple nonsulfur bacteria (Imhoff, 1988; Imhoff and Trüper, 1992). A suitable medium for growth of *Rhodoplanes* consists of (per liter of distilled water): (NH₄)₂SO₄, 1 g; KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.05 g; trace element solution SL12 (Pfennig and Trüper, 1992), 1 ml; yeast extract (Difco Laboratories), 1 g; 20 mM pyruvate (final concentration; filter-sterilized; pH 6.8).

Alternatively, a mineral medium supplemented with 0.2% tartrate and 0.1% succinate as carbon sources and niacin, thiamine, and *p*-aminobenzoic acid as growth factors is most effective for the enrichment of *Rhodoplanes*. The bacteria are enriched by anaerobic incubation in screw-capped test tubes filled with the medium under incandescent illumination. Direct plating or

membrane-filter plating techniques using anaerobic jars can also be used for isolation of *Rhodoplanes* species.

MAINTENANCE PROCEDURES

Cultures are well preserved in liquid nitrogen, by lyophilization, or at -80°C in a mechanical freezer.

DIFFERENTIATION OF THE GENUS *RHODOPLANES* FROM OTHER GENERA

The genus *Rhodoplanes* is phenotypically similar to other genera of the budding phototrophic bacteria, especially the genus *Rhodopseudomonas*, but is separated from the latter genus by its phylogenetic position and outstanding physiological and chemotaxonomic characteristics. Characteristic features of the genus *Rhodoplanes* are denitrification, inability to grow on sugars, long straight-chain fatty acids (>C₅), and sulfide, as well as production of both Q-10 and RQ-10. In addition, *Rhodoplanes* species are differentiated from the phenotypically similar *Rhodopseudomonas palustris* by cell size, vitamin requirement, and carbon nutrition. It is quite easy to distinguish *Rhodoplanes* from its nearest phylogenetic neighbors, the *Blastochloris* species, because the latter produce bacteriochlorophyll *b* and have either Q-9 and MK-9 or Q-8 (Q-10) and MK-7 (MK-8) as the major quinones. Major differentiating properties between *Rhodoplanes* species and other phototrophic *Alphaproteobacteria* are shown in Tables BXII.α.168 and BXII.α.169 of the chapter on the genus *Blastochloris* as well as in Tables 3 (pp. 125–126) and 4 (p. 127) in the introductory chapter “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A. The phylogenetic relationships of these bacteria, based on 16S rDNA sequences, are shown in Fig. 2 (p. 128) of that chapter.

TAXONOMIC COMMENTS

Rhodoplanes roseus was first described as *Rhodopseudomonas rosea* (Janssen and Harfoot, 1991). Later, this species was shown by 16S rDNA sequence analysis and chemotaxonomic information to be different at the generic level from any species of the genus *Rhodopseudomonas*. As a result, *Rhodopseudomonas rosea* was transferred to the new genus *Rhodoplanes* and designated as the type species of this genus, *Rhodoplanes roseus* (Hiraishi and Ueda, 1994b). At the same time, *Rhodoplanes elegans* was described as the second species of this genus. Furthermore, another isolate (strain IL245), classified as *Rhodopseudomonas* sp. (Kawasaki et al., 1993b), is closely related to the two *Rhodoplanes* species at a level of 97% sequence similarity of the 16S rDNA and may represent another species of *Rhodoplanes*. Phylogenetically, *Rhodoplanes* forms a major cluster in the *Alphaproteobacteria* together with the genus *Blastochloris* and the nonphototrophic genus *Ancylobacter*.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOPLANES*

Two species, *Rhodoplanes roseus* and *Rhodoplanes elegans*, currently comprise this genus. They are phenotypically quite similar to each other, but major differences are noted in budding type, rosette formation, vitamin requirement, and growth at pH 6.0. The average level of genomic DNA–DNA hybridization between

R. roseus and *R. elegans* is 37% (Hiraishi and Ueda, 1994b). The two species show approximately 98% sequence similarity of their 16S rDNA. General and differential characteristics of the two species are shown in Tables BXII.α.168 and BXII.α.169 of the chapter on the genus *Blastochloris*.

List of species of the genus *Rhodoplanes*

1. ***Rhodoplanes roseus*** (Janssen and Harfoot 1991) Hiraishi and Ueda 1994b, 671^{VP} (*Rhodopseudomonas rosea* Janssen and Harfoot 1991, 27.)
roseus L. adj. *roseus* rose-colored, pink.

Cells are rod-shaped to elongate-ovoid, 1.0 × 1.8–2.5 μm, motile by means of single polar flagellum. They reproduce by budding without prosthecae and do not form rosettes and clusters. If cells age, they become nonmotile

and encapsulated by slime. Internal photosynthetic membranes are present as lamellar stacks parallel to the cytoplasmic membrane. Phototrophically grown cultures are pink, while aerobic cultures are colorless. Absorption maxima of living cells are at 373–375, 468, 493, 527–530, 591–593, 799–801, and 850–854 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series.

Growth is preferably photoheterotrophic under anoxic conditions in the light. Photoautotrophic growth with thiosulfate, but not sulfide, occurs in the presence of 0.01% yeast extract. Chemotrophic growth is possible under oxic conditions in the dark at full atmospheric oxygen tension and by denitrification under anoxic conditions in the presence of nitrate.

Electron donors and carbon sources used for phototrophic growth are acetate, propionate, butyrate, valerate, lactate, pyruvate, succinate, fumarate, malate, citrate, tartrate, Casamino acids, yeast extract, and peptone. The following organic compounds are not utilized: formate, long-chain fatty acids with more than five carbon atoms, glycolate, malonate, benzoate, arabinose, xylose, rhamnose, fructose, glucose, mannose, galactose, dulcitol, mannitol, sorbitol, glycerol, methanol, ethanol, propanol, alanine, aspartate, asparagine, glutamate, and leucine. Sulfide is growth-inhibitory at 0.2 mM concentration and is not used as a photosynthetic electron donor. Ammonium salts are used as nitrogen sources. Sulfate and thiosulfate are assimilated as sulfur sources. Growth is also stimulated considerably by addition of 0.01% yeast extract. Niacin is required as growth factor.

Mesophilic freshwater bacterium with optimal growth at 30°C, pH 7.0–7.5 (pH range: 6.5–8.0). No growth occurs at pH 6.0 and 8.5 and at 1% NaCl.

Habitat: polluted freshwater lake sediments and wastewater environments.

Major quinone components are Q-10 and RQ-10.

The mol% G + C of the DNA is: 66.8 (HPLC) and 66.0 (T_m).

Type strain: 941, DSM 5909.

GenBank accession number (16S rRNA): D14429, D25313.

2. *Rhodoplanes elegans* Hiraishi and Ueda 1994b, 672^{VP}

e. elegans. L. adj. *elegans* choice, elegant.

Cells are rod-shaped, occasionally slightly curved, 0.8–1.0 × 2.0–3.5 µm, and motile by means of polar or subpolar flagella. They multiply by budding and asymmetric cell division, with a slender prostheca occurring between the mother and daughter cells. If cells age, they form rosette-like clusters. Internal photosynthetic membranes are present as lamellar stacks parallel to the cytoplasmic membrane. Phototrophically grown cultures are pink, while aerobic cultures are colorless. Absorption maxima of living cells are at 373–375, 466, 491, 527–530, 591–593, 799–801, and 854–856 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series.

Growth is preferably photoheterotrophic under anoxic conditions in the light. Photoautotrophic growth occurs with thiosulfate, but not sulfide, in the presence of 0.01% yeast extract. Chemotrophic growth is possible under oxic conditions in the dark at full atmospheric oxygen tension and by denitrification under anoxic conditions in the presence of nitrate.

The following organic compounds are photoassimilated: acetate, propionate, butyrate, valerate, caproate, lactate, pyruvate, succinate, fumarate, malate, citrate, tartrate, Casamino acids, and yeast extract. Not utilized are formate, long-chain fatty acids with more than six carbon atoms, glycolate, malonate, benzoate, arabinose, xylose, rhamnose, fructose, glucose, mannose, galactose, dulcitol, mannitol, sorbitol, glycerol, methanol, ethanol, propanol, alanine, aspartate, asparagine, glutamate, and leucine. Sulfide is growth-inhibitory at 0.2 mM concentration and is not used as a photosynthetic electron donor. Ammonium salts are used as nitrogen sources. Sulfate and thiosulfate are assimilated as sulfur sources. Thiamine and *p*-aminobenzoic acid are required as growth factors.

Mesophilic freshwater bacterium with optimal growth at 30–35°C and pH 7.0 (pH range: 6.0–8.5). No growth occurs at pH 5.5 or 8.5 or at 1% NaCl.

Habitat: activated sludge.

Major quinone components are Q-10 and RQ-10.

The mol% G + C of the DNA is: 69.6–69.7 (HPLC).

Type strain: AS130, ATCC 51906, JCM 9224.

GenBank accession number (16S rRNA): D25311.

Genus XVIII. *Seliberia* Aristovskaya and Parinkina 1963, 56^{AL}

JEAN M. SCHMIDT AND SUZANNE V. KELLY

Se.li.be'ri.a. M.L. fem. n. *Seliberia* of Seliber, named for the Russian microbiologist, Professor G.L. Seliber.

Rods, 0.5–0.8 µm in diameter and 1–12 µm in length, with a **helically sculptured** or **furrowed** topography. The ends of the cell may be either blunt or rounded. **Stellate aggregates (rosettes)** of **sessile rods** joined at one pole, and individual, shorter **motile rods (swarmers)** occur in the same culture. An adhesive **holdfast**, secreted at one pole, mediates attachment into rosettes. Growth on appropriate soil extract media may permit formation of round to ovoid “**generative cells**”. Capsules are not produced, although a thin glycocalyx is produced later in the growth cycle. Resting stages are not known. Gram negative. Following **unidirectional polar cell growth**, a shorter motile cell (a **swarmer**) and a longer sessile cell are produced by **asymmetric transverse fission**. A sin-

gle **subpolar ensheathed flagellum** is characteristically present on the swarmer; **several lateral flagella, not ensheathed**, may also be present. **Strictly aerobic**. Optimal temperature, 25–30°C; maximum ca. 37°C, minimum 15–20°C. Chemoorganotrophic, having an **oxidative type of metabolism**. Catalase and oxidase positive. These organisms live in soil and fresh water environments as autochthonous microflora, often where oligotrophic conditions prevail.

The mol% G + C of the DNA is: 63–66.

Type species: *Seliberia stellata* Aristovskaya and Parinkina 1963, 55.

FURTHER DESCRIPTIVE INFORMATION

Phylogenetic Treatment Limited phylogenetic information on the seliberias is available, due in large part to difficulties encountered in cell lysis for nucleic acid extractions. The analysis of 5S rRNA sequences has placed the genus *Seliberia* in the *Alphaproteobacteria* (Bulygina et al., 1990; Stackebrandt, 1992).

Cell morphology The typical cells are helically structured rods of varying lengths (Figs. BXII.α.236, BXII.α.239, and BXII.α.240). In a growing culture, the shorter cells are motile swimmers; the longer cells are sessile and include the predivisional cells. The rods become very long (10 μm or more in length) when grown on fulvic acid medium (Aristovskaya, 1974); in aquatic environments with very dilute organic nutrient conditions, seliberias also occur as very long helically sculptured rods. In media containing ulmic acid complexes (Aristovskaya, 1974; Schmidt and Swafford, 1979) or when a plentiful supply of organic nutrients is available (Schmidt and Swafford, 1979), the cells are shorter, and the longest cells seldom exceed 5 μm in length. Growth of some strains of *Seliberia stellata* on soil extract media (Aristovskaya and Parinkina, 1963; Aristovskaya, 1964, 1974) may give rise to round or ovoid "generative cells"; production of the round to ovoid generative cells with the type strain of *S. stellata* (ICPB 4130) has not been verified (Schmidt and Starr, 1984).

Cell wall composition The cell wall of *Seliberia stellata* is a bipartite structure with an outer layer of tightly bound protein, but lacking the appearance of an S layer. The outer protein layer is removed from the underlying layer of peptidoglycan with 0.5% KOH, but not with a variety of milder enzymatic and/or detergent treatments. No phospholipid or lipopolysaccharide (LPS) has been found in *Seliberia* cell wall preparations.

Fine structure Ultrastructural features of *Seliberia stellata* are shown in Fig. BXII.α.237.

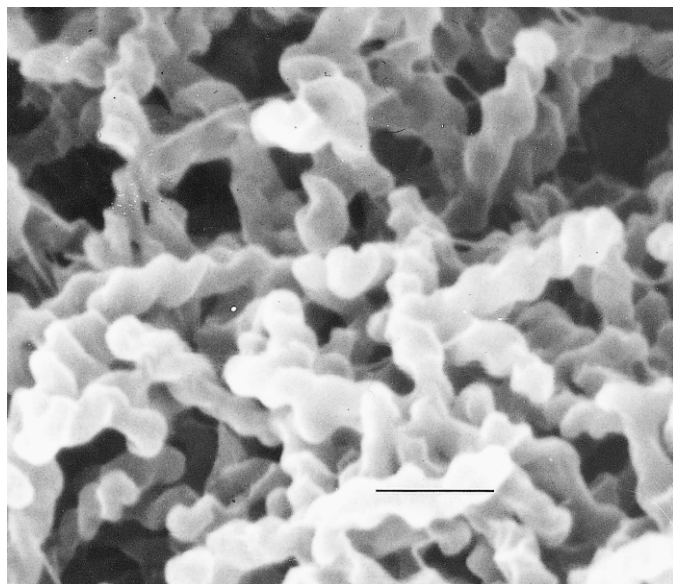


FIGURE BXII.α.236. Scanning electron micrograph of *S. stellata* strain Z/A fixed with 2.0% glutaraldehyde, critical-point dried, and gold-coated, showing the helically sculptured topography of the bacteria. Bar = 1 μm (Courtesy of J.R. Swafford).

Colonial and cultural characteristics On dilute peptone-yeast extract agar, colonies are slow-growing, requiring a week or more of incubation. The colonies are minute (0.5–1.0 mm diameter) and white in color. The colonies may adhere strongly to the agar. On ulmic acid or fulvic acid media, the colonies are also slow-growing and may show some browning, due to presence of iron oxide. In dilute liquid peptone-yeast extract medium, the bacteria adhere tightly to the surface of the growth vessel, and adherence is mediated by the polar holdfast.

Life cycle Cell growth occurs unidirectionally, at the pole of the rod opposite the pole with the secreted holdfast. The end of the predivisional rod that consists of the newly made surface components eventually becomes a motile swimmer as a result of an asymmetric division. The remaining (longer) portion of the predivisional cell retains the surface components of the parent without redistributing them into the zone of new growth. The



FIGURE BXII.α.237. Thin section of *S. stellata* strain Z/A, prepared with a modification of the Ryter-Kellenberger fixation method (Kellenberger et al., 1958), 1.0% osmic acid in 0.1× buffer for 2 h, and embedded in Spurr resin. The thin cell envelope and wavy outline are characteristic of seliberias. Bar = 0.1 μm (Courtesy of J.R. Swafford).



FIGURE BXII.α.238. Nomarski interference contrast light micrograph of *S. stellata* rosettes. Bar = 5 μm (Courtesy of J.R. Swafford).

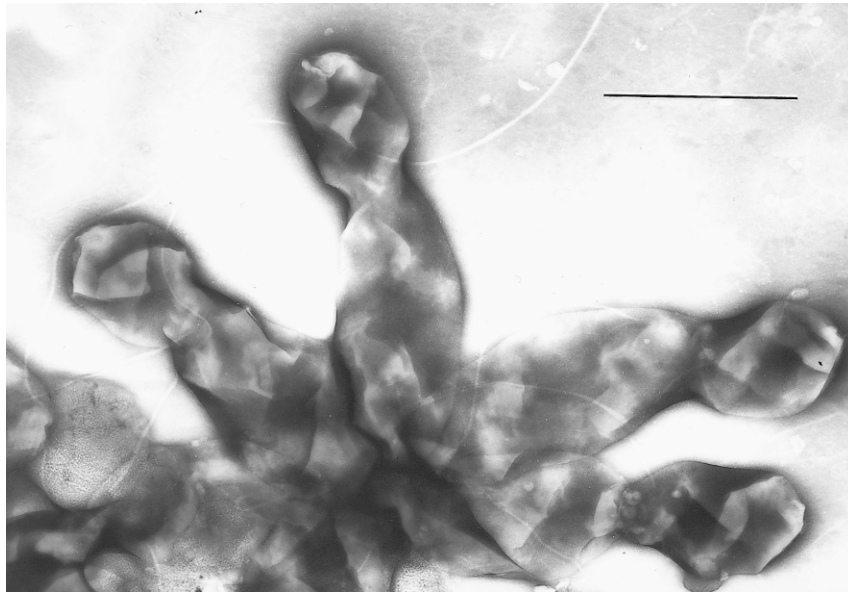


FIGURE BXII.α.239. Phosphotungstate (0.5%) negative stain of an aquatic *Seliberia*-like strain, ICPB 4141, illustrating the asymmetric plane of division. Bar = 1.0 μm.

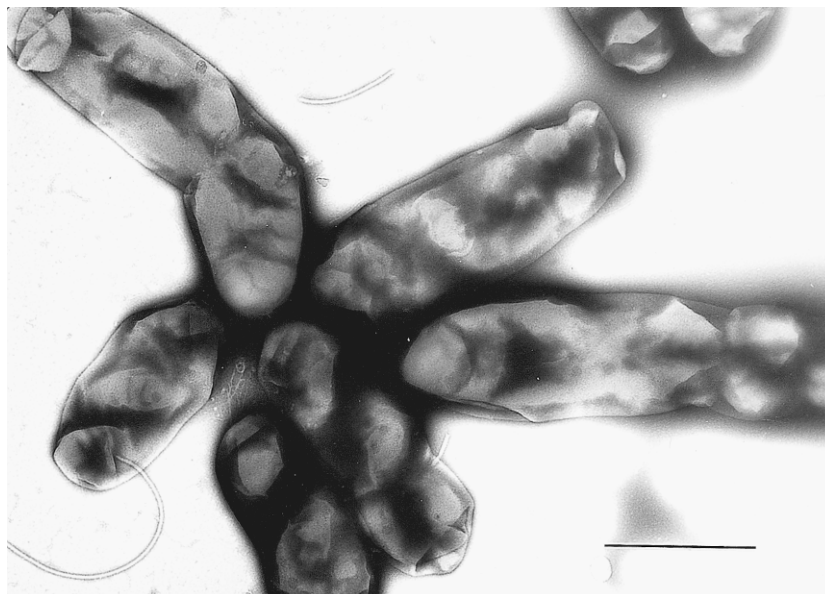


FIGURE BXII.α.240. Phosphotungstate (0.5%) negative stain of a rosette of *Seliberia*-like aquatic strain ICPB 4133. A subpolar flagellum is present. Bar = 1.0 μm (Courtesy of J.R. Swafford).

subpolar ensheathed flagellum of the new swarmer is found at the apical end of the predivisional cell, shortly before its division. The asymmetry perpendicular to the division plane results in unequal division products (Fig. BXII.α.239; Schmidt and Starr, 1984). Swarmer cell production by unidirectional polar growth in the genus *Seliberia* meets two of the major criteria (*de novo* synthesis of the bud surface and transverse asymmetry of division) of accepted definitions of budding (Staley, 1973a; Staley et al., 1981); however, a third feature typical of most budding bacteria, an increasing diameter of the bud during its development (Hirsch, 1974a), is not found in *Seliberia*; the developing daughter cell (swarmer) is (i) not initially narrower than the parent, and

(ii) does not increase in diameter (width) during growth (Schmidt and Starr, 1984).

The proportion of cells in stellate aggregates (rosettes) varies in *Seliberia* cultures, but rosettes are readily detectable microscopically under most cultural conditions (Figs. BXII.α.238, BXII.α.240). The stellate aggregates are formed by association of swimmers, which have produced an adhesive holdfast at one pole, with sessile cells—often with those stationary rods located in the immediate vicinity of the predivisional cell that produced the swarmer. In addition, at high cell densities in liquid cultures, polar aggregation of swarmer cells occurs (Schmidt and Starr, 1984), akin to the manner of formation of *Caulobacter* rosettes

(Poindexter, 1964, 1981). The *S. stellata* holdfast consists of a polysaccharide, containing glucose and/or mannose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (Hood and Schmidt, 1996).

Nutrition and growth conditions Growth of *Seliberia* occurs on dilute organic media, such as yeast extract plus peptone or Casamino acids (Difco), soil extract agar, or pond water agar. Media containing organomineral complexes of either fulvic or ulmic acid with sesquioxides have been used (Aristovskaya and Parinkina, 1963; Aristovskaya, 1974; Schmidt and Swafford, 1981). With these media, aerobic incubation at 25°C gives satisfactory growth.

Metabolism and metabolic pathways Although the genus *Seliberia* has been previously described as facultatively anaerobic (Aristovskaya, 1974), the available type strain (Z/A=ICPB 4130) and all of the several aquatic strains are strictly oxidative; all lack the capacity to carry out anaerobic fermentations. *S. stellata* Z/A possesses glucose dehydrogenase and 6-phosphogluconate dehydrase activities, and can grow aerobically on D-glucuronic acid, suggesting the presence of the Entner–Doudoroff pathway. Strain Z/A also possesses strong NADH oxidase activity, isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase, consistent with oxidative metabolism. *S. stellata* strain Z/A is a strong denitrifier and can grow anaerobically in the presence of 0.1% NaNO₃, indicating a nitrate/nitrite respiration. Most of the aquatic *Seliberia*-like strains are not able to denitrify anaerobically (Schmidt and Swafford, 1979, 1981).

Genetics and molecular data The seliberias have not received much attention, due to difficulties in obtaining DNA preparations, which are related to the problems encountered in obtaining cell lysis. No bacteriophages or plasmids have been reported for the seliberias.

Antigenic structure The outer layer of the cell envelope of the seliberias induces an antibody response in rabbits (Schmidt and Starr, 1984). Indirect fluorescent antibody and agglutination tests done with polyvalent antisera to strain Z/A and to two aquatic *Seliberia*-like strains indicated significant antigenic relatedness among the strains. The two major proteins making up the outer layer of strain Z/A (which lacks LPS) have respective molecular weights of 54–56 kDa and 82–84 kDa. Some information on flagellum-associated proteins is available: strain Z/A, which produces both ensheathed subpolar and nonensheathed lateral flagella, has flagella-associated proteins with molecular weights of 68, 36, and 35 kDa; several aquatic *Seliberia*-like strains which produced only the subpolar ensheathed flagellum gave major flagellar protein values of 56–60 kDa.

Antibiotic Sensitivity Seliberias are quite resistant to low concentrations (10 µg) of most of the common antibiotics; most aquatic *Seliberia*-like strains are inhibited by 100 µg concentrations of ampicillin, penicillin G, vancomycin, chlortetracycline, streptomycin, and rifampin. *S. stellata* strain Z/A is resistant to 100 µg concentrations of ampicillin, penicillin G, and vancomycin.

Pathogenicity No reports of seliberias causing overt or opportunistic infections in humans, animals, or insects have been reported. Their optimal growth temperature range of 25–30°C, poor growth at 37°C, and lack of LPS in their cell wall makes the seliberias unlikely candidates as pathogens of humans or warm-blooded animals.

Ecology The habitats of *Seliberia* include soil and freshwater environments. They have been reported as epiphytes of the cyanobacterium *Nodularia* (Smarda, 1985), and have been found in an activated sludge reactor (Baker et al., 1983). Although seliberias appear to be widely distributed, they have not received much attention from bacteriologists. Seliberias appear to be well suited to conditions of limited nutritional resources, although they can also occur in eutrophic environments.

ENRICHMENT AND ISOLATION PROCEDURES

Specific enrichment procedures for podzol-inhabiting seliberias have not been described, although it has been noted that these bacteria occur frequently in pedoscopes (Aristovskaya and Parinkina, 1963). Seliberias of both aquatic and soil origin can be enriched, nonspecifically, using oligotrophic conditions (Schmidt and Swafford, 1979, 1981). Glass beakers, to which water samples (for example, 200–800 ml of pond, lake, or even tap water) are added, with or without the addition of 0.001–0.005% peptone (Difco), are covered with plastic film to prevent evaporation and incubated for several days to several weeks or months at 24–26°C. To adapt this aquatic enrichment to soil inocula, suspensions of 1 g (or less) of soil are made in 500 ml of filtered pond or tap water to which (i) no nutrients or (ii) very low concentrations of peptone are added (as above, for aquatic sample enrichment). The seliberias usually occur at the air–water interface of the enrichment (in the surface pellicle, if heavy growth has occurred). They may be attached to other microbes or detritus. Seliberias will be easier to isolate subsequently if heavy growth of other, more rapidly growing bacteria has not occurred in the enrichment beaker. Hence, the use of several enrichment beakers with varying concentrations of added nutrients may facilitate the enrichment procedure. Observation of the bacteria at the surfaces of the enrichment, using transmission electron microscopy and negative stains (Schmidt and Swafford, 1992) is useful for determining whether significant numbers of seliberias are present. Use of genus-specific fluorescent dye-labeled oligonucleotide probes in glass slide hybridization reactions for these bacteria has not been reported yet, but it should be feasible once sequences are available.

To obtain pure cultures of seliberias, several types of solid culture media have been used. To isolate seliberias from soil samples, agar media containing dilute organomineral complexes (ulmic or humic acids, or fulvic acid) obtained from soil humus (Aristovskaya and Parinkina, 1961, 1963) have been used successfully. Soil samples thought to contain seliberias are streaked on the ulmic or fulvic acid agar media; after 4–7 d, the slow-growing colonies that show some ferric oxide–caused browning are examined for characteristic morphologic features. The accumulation of ferric hydroxide by seliberias of soil origin is characteristic of their behavior in mixed culture; in pure culture, an atmosphere of about 1% CO₂ stimulates deposition of iron oxide (Aristovskaya and Parinkina, 1963).

Seliberia-like bacteria of aquatic or soil origin may be isolated (from enrichment cultures) by streaking from the liquid enrichment surface onto a dilute organic medium; recipes for two useful media follow. PYE medium: 0.2% peptone (Difco), 0.1% yeast extract, 1.0% Hutner's vitamin-free mineral base (Cohen-Bazire et al., 1957), 1.2% or 1.5% Bacto-agar (Difco), and distilled water. Dilute medium I: 0.02% peptone, 0.01% yeast extract, 1.0% Hutner's mineral base, 1.2 or 1.5% Bacto-agar (Difco), and distilled water; addition of 0.1% glucose (filter-sterilized) is optional (Schmidt and Swafford, 1981). After two weeks or longer of in-

cubation at 25°C, the plates are examined with a dissecting microscope for minute 0.5–1.0 mm diameter white colonies that may adhere to the agar. The selected colonies are transferred to patch plates, reincubated for several days, and then observed with phase-contrast light microscopy for rosette-forming, asymmetrically dividing, rod-shaped bacteria that have a motile stage. Transmission electron microscopy, using negative stains, can be used to check for the helical sculptured topography of the rods (Schmidt and Swafford, 1981). Specific oligonucleotide probes with fluorescent labels may be useful in identification of *Seliberia* colonies or patches.

MAINTENANCE PROCEDURES

Stock cultures grown aerobically on slants of PYE medium in screw-cap test tubes remain viable for several weeks at room temperature (24–26°C). The slant cultures survive several weeks longer at 4°C. Suspensions of cells scraped (with some difficulty) from agar surfaces and suspended in sterile distilled water also survive well for several weeks at room temperature. For longer-term preservation (several years), lyophilization is a satisfactory method.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

The determination of cellular morphology at a level of resolution attainable by scanning electron microscopy or transmission electron microscopy (negative stains) is a key feature in the identification of seliberias. The helical sculpturing of the bacterial cell surface and the presence of a subpolar sheathed flagellum can be determined with electron microscopy.

DIFFERENTIATION OF THE GENUS *SELIBERIA* FROM OTHER GENERA

At the resolution achievable with light microscopes, seliberias can be confused with various kinds of caulobacters (members of the genera *Caulobacter* or *Asticcacaulis*), because of their occurrence in the same types of natural habitats and preference for aerobic environments, similarities in cell size and apparent rod-like shape, presence of shorter motile and longer sessile cells, and occurrence of rosette arrangements (star-like aggregates) of several to many cells. However, seliberias are not prosthecate, whereas predivisional and mature *Caulobacter* and *Asticcacaulis* cells have at least one prostheca or cellular stalk (Poindexter, 1964, 1981). Prosthecae can be observed with phase contrast high-resolution light microscopy but not without some difficulty

since prosthecae are usually 0.2 µm or less in diameter. Other rosette-forming bacteria, which also demonstrate motility, but which typically belong to the genera *Pseudomonas* or *Agrobacterium*, also can be difficult to distinguish from seliberias if only light microscopy is used.

Confirmation that an isolate belongs to the genus *Seliberia* can be established by phenetic characterization, although use of morphological and ultrastructural characteristics as a sole guide to generic assignment is not a desirable situation. Notably, there is still some lack of phylogenetic data for the seliberias, but obtaining phenetic information on the physiology, relationship to oxygen, mol% G + C, and mode of growth and division is useful in defining members of the genus *Seliberia*, distinguishing them from *Caulobacter*, pseudomonads, *Agrobacterium* spp., and other rosette-forming Gram-negative bacteria (Table BXII.α.188). Since the seliberias characteristically exhibit unidirectional polar growth, and their swimmers are generated at the end of the dividing cell (Schmidt and Starr, 1984), this relatively unusual mode of cell division is a useful distinguishing trait.

TAXONOMIC COMMENTS

The genus *Seliberia*, its single species *Seliberia stellata*, and the closely related but not quite identical aquatic *Seliberia*-like strains have not been examined for 16S rDNA sequences; 5S rRNA analysis has placed this genus in the *Alphaproteobacteria* (Bulygina et al., 1990; Stackebrandt, 1992). Difficulties in achieving cell lysis and DNA extractions have played a role in the delay in acquiring 16S rDNA phylogenetic data and DNA–DNA hybridization data among the phenetically quite similar *Seliberia*-like strains. The DNA–DNA hybridization data among *Seliberia*-like strains are important in determining species-level relationships. Seliberias also should be compared at the molecular level with other *Alphaproteobacteria*, including various species of *Caulobacter*, some pseudomonads, such as *Pseudomonas carboxydohydrogena* (Meyer et al., 1980) due to its morphology, and *Nitrobacter*, a budding bacterium with an unusual cell envelope similar in profile to that of *Seliberia stellata*, to establish the uniqueness of this genus.

ACKNOWLEDGMENTS

We thank G.A. Zavarzin, Institute of Microbiology, Academy of Sciences, Moscow, for the culture of *Seliberia stellata* strain Z/A (ICPB 4130). Micrographs were provided by J.R. Swafford. Data on cell wall composition and metabolic pathways were obtained in collaboration with Steven Baker and Michelle Santos.

TABLE BXII.α.188. Differential morphological and developmental characteristics of *Seliberia*^a and other biochemically and morphologically similar taxa^b

Characteristic	<i>Seliberia</i>	<i>Agrobacterium</i>	<i>Caulobacter</i>	<i>Pseudomonas</i>
Unidirectional polar cell growth	+	—	—	—
Asymmetric cell division products	+	—	+	—
Ensheathed polar flagellum	+	—	—	D
Prostheca production	—	—	+	—
Cell surface helically sculptured	+	—	—	— ^c
Stellate aggregate formation	+	D	+	D
Mol% G + C of DNA	63–66	58–62	62–67 ^d	58–70 ^e

^a*S. stellata* and the aquatic and soil *Seliberia*-like strains.

^bSymbols: +, 90% of strains positive; —, 90% of strains negative; D, different reactions in different species.

^cData from Meyer et al., 1980.

^dData from Poindexter, 1981.

^eData from Bergan, 1981.

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List of species of the genus *Seliberia*

1. ***Seliberia stellata*** Aristovskaya and Parinkina 1963, 55^{AL}
stel.la' ta. L. adj. *stellata* starred.

Colonies on peptone-yeast extract (PYE medium, see Enrichment and Isolation Procedures, above) are smooth, convex, and white. The diameter is 0.5–1.5 mm, with a regular border. On ulmic acid agar, a soil extract medium (Aristovskaya and Parinkina, 1961, 1963), growth is sparse, colonies are less than 1.0 mm in diameter, and light brown. *Seliberia stellata* strain Z/A grows in a chemically defined medium containing an appropriate carbon source, 0.05% ammonium chloride, 1.0% Hutner's vitamin-free mineral base (Cohen-Bazire et al., 1957), and 0.001% phosphate. It grows over a wide pH range, from 4.5 to at least 9.0. No

added vitamins are required. *S. stellata* is positive for urease production and starch hydrolysis; it can produce H₂S from cystine; it can reduce nitrate to nitrite under either aerobic or anaerobic conditions; it is unable to liquefy gelatin, utilize citrate, hydrolyze casein, or produce indole, and it gives negative methyl red and Voges-Proskauer reactions. *S. stellata* strain Z/A, the reference strain, can produce both a subpolar, ensheathed, typically single flagellum, and several lateral, ordinary (not ensheathed) flagella. This flagellation is characteristic of its swarmer cells grown on agar-solidified PYE medium at 25°C.

The mol% G + C of the DNA is: 66 (Bd) (strain Z/A).

Type strain: E-37, VKM B-1340.

Other Organisms

Several *Seliberia*-like strains from aquatic (freshwater) sources and a few from soil have been enriched and isolated in pure culture, and these are quite similar to *S. stellata*. Ten strains have been characterized in some detail. A typical aquatic *Seliberia*-like strain (ICPB 4133) is shown in Fig. BXII.α.240. Their description is the same as that for the genus, with the following additional characteristics: the aquatic *Seliberia*-like strains are unable to use any of 60 tested compounds as their sole carbon source; they appear to have complex growth factor requirements. They can be grown on the minimal medium described for *Seliberia stellata* supplemented with 0.001% Vitamin Assay Casamino Acids (Difco) and 0.1% (v/v) Staley's vitamin solution (Staley, 1968). They grow dependably but sparsely on PYE medium (see Enrichment and Isolation Procedures, above; Schmidt and Swafford, 1981).

Growth of aquatic *Seliberia*-like strains is very sparse on all complex media tested. Grown on PYE agar, colonies are white, minute (0.5–1.0 mm diameter), and very adherent to the agar medium. Growth in liquid PYE medium is granular, definitely not well dispersed, with a strong tendency for attachment to the submerged surfaces of glass culture vessels, so long as a shallow (about 1 cm deep) level of liquid medium is maintained. (The

growth of *S. stellata* under similar circumstances is well dispersed, with some rosettes visible microscopically, but without the presence of macroscopic granules). The aquatic *Seliberia*-like strains demonstrate an obligate requirement for oxygen and they reduce nitrate under aerobic conditions, but not anaerobically. They do not produce H₂S from cystine. Most of the *Seliberia*-like strains can hydrolyze urea, and all can hydrolyze starch.

All of the aquatic strains produce swimmers with a subpolar ensheathed flagellum, but most have not been found to produce the lateral, ordinary (i.e., not ensheathed) flagella. Two of the aquatic strains, which have been examined with indirect immunoferritin surface labeling during growth, were found to exhibit unidirectional polar cell surface growth, with production of swimmers having *de novo* synthesized surface antigens. *Seliberia stellata* exhibited the same kind of unusual growth and division pattern in similar experiments (Schmidt and Starr, 1984). Nine of 10 aquatic *Seliberia*-like strains cross-react moderately to strongly with *S. stellata* in serological tests (tube agglutination and indirect immunofluorescence tests). The aquatic seliberias are less resistant to common antibiotics than is *S. stellata*.

The mol% G + C of the DNA is: 63–65 (Bd) (aquatic *Seliberia*-like strains).

Genus XIX. ***Starkeya*** (ex Starkey 1934) Kelly, McDonald and Wood 2000, 1800^{VP}

DONOVAN P. KELLY AND ANN P. WOOD

Star.ke' ya. M.L. n. *Starkeya* of Starkey; named after Robert L. Starkey, who made important contributions to the study of soil microbiology and sulfur biochemistry.

Short rods, coccoidal or ellipsoidal cells 0.4–0.8 × 0.8–2.0 μm, occurring singly and, occasionally, in pairs. Nonmotile. Colonies grown on thiosulfate agar (with biotin) are small, smooth, circular, round, and opalescent, becoming white with sulfur. Thio-sulfate liquid medium (lacking biotin) becomes turbid and sul-

fur precipitates during static incubation; thiosulfate is incompletely used, and the pH falls from 7.8 to 5.8. This poor development is due to the requirement for biotin exhibited by the type strain. The organism is **facultatively chemolithoautotrophic**, but optimal autotrophic development requires biotin, and op-

timal heterotrophic growth requires yeast extract, biotin, or other additions, such as pantothenate, depending on the organic substrate. Autotrophic growth is also observed with formate when high levels of ribulose 1,5-bisphosphate carboxylase are expressed. Some strains may degrade methylated sulfides. This organism is **strictly aerobic, both autotrophically and heterotrophically**, and is incapable of denitrification. **Oxidizes and grows on thiosulfate and tetrathionate but not on sulfur or thiocyanate.** Ammonium salts, nitrates, urea, and glutamate are used as nitrogen sources. Optimal temperature 25–30°C; growth range 10–37°C (no growth at 5°C or 42°C). Optimal pH 7.0; growth range pH 5.7–9.0. Contains ubiquinone Q-10. Major cellular fatty acids are octadecenoic acid and cyclopropane acid of C₁₀; lacks a major hydroxy fatty acid. The lipopolysaccharide lacks heptoses and has only 2,3-diamino-2,3-dideoxyglucose as the backbone sugar. Member of the *Alphaproteobacteria*. Isolated from soil. Presumably widely distributed.

The mol% G + C of the DNA is: 67.3–68.4.

Type species: *Starkeya novella* (Starkey 1934) Kelly, McDonald and Wood 2000, 1800 (*Thiobacillus novellus* Starkey 1934, 365.)

FURTHER DESCRIPTIVE INFORMATION

This newly created genus currently contains only one of the former *Thiobacillus* species, *T. novellus*, a facultatively heterotrophic and chemolithoautotrophic, rod-shaped organism in the

Alphaproteobacteria. The properties, taxonomy, and differentiation of the genera of sulfur-oxidizing, chemolithoautotrophic, Gram-negative, rod-shaped bacteria are summarized in Tables BXII.β.70 and BXII.β.71 and Fig. BXII.β.60 in the chapter on the genus *Thiobacillus* in this *Manual*. *Starkeya* has been shown by 16S rRNA gene sequence analysis and comparison of physiological properties to not be a species of *Paracoccus* (which contains the physiologically comparable species *P. versutus* and *P. pantotrophus*), but rather to be significantly related only to *Ancylobacter* (Kelly et al., 2000). The description given here is based primarily on studies with the type strain, isolated from soil in the 1930s (Starkey, 1934, 1935a, b; Kelly et al., 2000). Another strain, isolated from sewage, has been shown to oxidize methanethiol, dimethylsulfide, and dimethyldisulfide (Cha et al., 1999), properties not yet reported for the type strain. Detailed studies of inorganic sulfur-compound oxidation have been undertaken with *S. novella*, including studies of its unique sulfite:cytochrome *c* oxidoreductase (Southerland and Toghrol, 1983; Toghrol and Southerland, 1983; Kappler et al., 2000) and its thiosulfate-oxidizing system. The latter was initially thought to be a multienzyme complex located exclusively in the cytoplasmic membrane system (Oh and Suzuki 1977), but Kappler et al. (2001) have shown that a periplasmic thiosulfate-oxidizing system not involving a membrane-anchored multienzyme system may also function (C. Dahl, personal communication).

List of species of the genus *Starkeya*

1. *Starkeya novella* (Starkey 1934) Kelly, McDonald and Wood 2000, 1800^{VP} (*Thiobacillus novellus* Starkey 1934, 365.) *no. vel' la*. L. dim. adj. *novella* new.

The species description is the same as the genus description, with the exception that oxidation of methylated sulfides has not been tested with the type strain. Biotin is required by the type strain for good growth on most sub-

strates; yeast extract may be substituted for biotin and, in some cases, the biotin requirement may be replaced by lipoic acid or coenzyme A; good growth on methanol requires pantothenate or yeast extract rather than biotin.

The mol% G + C of the DNA is: 67.3–68.4 (Bd, *T_m*).

Type strain: ATCC 8093, DSM 506, IAM 12100, IFO 12443, NCIMB 9113.

GenBank accession number (16S rRNA): D32247.

Genus XX. *Xanthobacter* Wiegel, Wilke, Baumgarten, Opitz and Schlegel 1978, 573^{AL}

JÜRGEN K.W. WIEGEL

Xan.tho.bac' ter. Gr. adj. *xanthos* yellow; M.L. masc. n. *bacter* the equivalent of Gr. neut. n. *bacterion* rod, staff; M.L. masc. n. *Xanthobacter* yellow rod.

Cells are rod shaped, sometimes twisted, 0.4–1.0 × 0.8–6.0 μm. Pleomorphic cells are produced on media containing tricarboxylic cycle-intermediates (especially succinate), whereas coccoid cells as well as cells up to 10 μm long are produced on media containing an alcohol as the sole carbon source. Refractile (polyphosphate) and lipid (poly-β-hydroxybutyrate) bodies are evenly distributed in the cells. Resting stages are unknown. Key sporulation genes are absent. Depending on the species and growth conditions, the cells are nonmotile or motile (by peritrichous flagella). The Gram reaction frequently appears falsely to be positive or variable due to polyphosphate granules; however, ultrastructurally and biochemically, the cell wall is of the negative Gram-type.¹ Aerobic, having a strictly respiratory type of metab-

olism with oxygen as the terminal electron acceptor. Optimal temperature, 25–30°C. Optimal pH, 5.8–9.0. Colonies are opaque and slimy (although “slimeless” strains exist) and are yellow due to a water-insoluble carotenoid pigment (zeaxanthin dirhamnoside). The color intensity depends on the amount of slime produced by individual strains. Catalase positive. All strains can grow chemolithoautotrophically in mineral media under an atmosphere of H₂, O₂, and CO₂ (7:2:1, v/v) as well as chemoorganoheterotrophically on methanol, ethanol, n-propanol, n-butanol, and various organic acids as sole carbon sources. The carbohydrate utilization spectrum is limited, and neither volatile/nonvolatile fatty acids nor gas is produced from carbohydrates such as fructose, glucose, or mannose. Some strains require vitamins. Some strains can utilize substituted thiophenes as sole carbon, energy, and sulfur sources. When degrading aliphatic epoxides, tested strains contain coenzyme M, which otherwise is a typical coenzyme of the obligate anaerobic methanogenic archaea. N₂ is fixed in nitrogen-deficient media under heterotrophic or chemolithoautotrophic growth conditions, but by most strains

1. The terms “Gram-type negative” and “Gram-type positive” were proposed by Wiegel (1981) to describe bacteria according to ultrastructural and biochemical Gram characteristics (cell wall structure, presence or absence of indicator compounds such as lipopolysaccharide; Wiegel and Quandt, 1982). The terms are distinct from those referring to the results of the Gram-staining reaction: “Gram-reaction positive”, “Gram-reaction negative”, or “Gram-reaction variable”.

only under a decreased O₂ pressure. The bacteria occur free-living in freshwater (mainly *X. agilis*), wet soil containing decaying organic material (*X. autotrophicus*, *X. flavus*), marine sediments (*X. flavus*), compost of root balls of *Tagetes* (thiophen-utilizing *X. tagetidis*), and associated with the roots of plants including wetland rice (*X. flavus*). *Xanthobacter* can be regarded an associative N₂-fixing bacterium (rice, tagetis, coconut palm). The induction of root or stem nodules has not been observed. 16S rDNA sequence analysis places the members into the class *Alphaproteobacteria*; however, the presently recognized species of *Xanthobacter* are intermixed with the single-species genera *Aquabacter* and *Azorhizobium*, and together they form a distinct cluster.

The mol% G + C of the DNA is: 65–70 (*T_m*) and 66–68 (Bd).

Type species: *Xanthobacter autotrophicus* (Baumgarten, Reh and Schlegel 1974) Wiegeler, Wilke, Baumgarten, Opitz and Schlegel 1978, 580 (*"Corynebacterium autotrophicum"* Baumgarten, Reh and Schlegel 1974, 214.)

FURTHER DESCRIPTIVE INFORMATION

Colonial characteristics All species of *Xanthobacter* form colonies that are smooth, convex, circular, filiform, opaque, and either yellow, off-white, or—when abundant slime is produced—nearly colorless. Due to the slime formation, especially on nutrient broth agar plates, colonies develop into characteristic colony forms called "fried egg". The main component of the yellow pigment of all strains (the "white" strains contain just minor amounts) has been identified as zeaxanthin dirhamnoside (Hertzberg et al., 1976; J. Wiegeler and K. Schmidt, unpublished data). It is water insoluble and is located in the cell wall (Eberhardt, 1971). In *Aquabacter* and *Azorhizobium*, this pigment has not been identified so far; their colonies are creamy white.

Cell morphology The cell shape of *Xanthobacter* depends strongly on the growth conditions, i.e., especially the carbon source, whereas the nitrogen source has a minor effect (Fig. BXII.α.241). Cells under N₂-fixing conditions are only slightly longer than in the presence of ammonia or other sources of combined nitrogen. Cells of both *X. autotrophicus* and *X. flavus* are coccoid when growing on *n*-propanol (Fig. BXII.α.241) and are long, filamentous rods when growing on *n*-butanol. One of the most significant morphological features is the formation of irregular, twisted (especially *X. tagetidis*) (Padden et al., 1997), and branched cells (especially *X. autotrophicus* and *X. flavus*) during growth on tricarboxylic acid cycle intermediates; the intermediates include succinate, which is a good growth substrate and promotes the development of the most irregular cell forms during the late exponential growth phase (Fig. BXII.α.241) (Wiegeler and Schlegel, 1976). In contrast to strains of *X. autotrophicus*, *X. flavus*, and *X. tagetidis*, the strains of *X. agilis* (motile under nearly all growth conditions) are only slightly pleomorphic and exhibit branched cell formation only after prolonged incubation (3–7 d) on succinate-containing agar media (Aragno et al., 1977; J. Wiegeler, unpublished data). Electron micrographs of ultrathin sections show that the branching cells do not contain septa at the branching points (Fig. BXII.α.242) (Wiegeler et al., 1978). Scanning electron micrographs from *X. tagetidis* reveal twisted, nearly fusiform (i.e., with tapered ends) spirals (Padden et al., 1997). The occurrence of a snapping type of cell division, as was assumed from light-microscopic inspection of *X. autotrophicus*, has not been confirmed by electron-microscopic studies. The illusion of snapping cell division, as well as of "star formation" (Fig. BXII.α.241), is presumably due to cell aggregation by copious

amounts of slime. Surface patterns on the outer cell wall are not observed in electron micrographs. The cells of the exponential growth phase and those grown under heterotrophic conditions are usually larger than those of the early stationary growth phase and those grown under chemolithoautotrophic conditions; the latter are mainly straight or only slightly curved rods (Fig. BXII.α.241).

Cell wall type and Gram-reaction Many strains identified later as strains of *Xanthobacter* were originally described to give a positive or variable Gram reaction (Baumgarten et al., 1974). However, *Xanthobacter* belongs to the negative Gram-type bacterial group, since it contains lipopolysaccharides (Fig. BXII.α.243) (Wiegeler and Mayer, 1978; Wiegeler, 1981). The impression of a positive Gram-staining reaction is feigned by the content of refractile bodies, which were identified as polyphosphate granules by electron microscopy, as well as by volutin staining (Figs. BXII.α.242 and BXII.α.243). The polyphosphate content of the cells is high (15 mg of phosphate/g dry weight) and even at a low phosphate concentration in the growth medium (0.01 mM) volutin granules do not disappear (Wiegeler et al., 1978). Using phosphate limitation or starvation, attempts to obtain cells of *X. autotrophicus* without polyphosphate granules have failed. Large dark bodies, frequently visible in the light microscope, are located at one or both (or in branched cells on several) ends of the cells. Electron microscopy of thin sections of cells (Walther-Mauruschat et al., 1977) indicated that these larger granules are poly-β-hydroxybutyrate granules (Fig. BXII.α.242).

The cell envelope of *X. autotrophicus* has a thin multilayered structure, resembling that of Gram-type negative cells (Fig. BXII.α.242) (Walther-Mauruschat et al., 1977). The peptidoglycan content of the cell wall of the *X. autotrophicus* strains (15–25%) is intermediate compared to that of typical Gram-type positive (30–70%) and Gram-type negative (10%) bacteria. Teichoic acid and teichuronic acid are absent (Schleifer and Kandler, 1972; O. Kandler and F. Fiedler, personal communication), but elevated concentrations of glycine can be present (see below for details).

Compared to other Gram-type negative bacteria the lipopolysaccharide content is relatively low for the strains tested. The lipopolysaccharide was identified both chemically after extraction from autotrophic cells and by the lipopolysaccharide polymyxin B interaction technique using electron microscopy (Wiegeler and Mayer, 1978; Wiegeler, 1981) (Fig. BXII.α.243). The citrate synthase of *Xanthobacter* species resembles that of Gram-type negative bacteria by having a molecular weight above 250,000 and by being inhibited by NADH (Weitzman and Jones, 1975; Berndt et al., 1976; Weitzman, 1987). All these properties are in agreement with the 16S rDNA sequence analysis placing *Xanthobacter* in the class *Alphaproteobacteria* (Rainey and Wiegeler, 1996).

Slime formation The majority of the strains produce copious amounts of slime (Fig. BXII.α.244), which consists mainly of glucose, galactose, mannose, and uronic acid (unidentified; 30% or more of the monomers contain a carboxyl group (Andreesen and Schlegel, 1974; Opitz, 1977). At high C:N ratios, the slime formed can cause gelatinization and solidification of the medium without a significant increase in cell mass. The amount of slime varies with the strain as well as growth conditions. Most strains produce copious amounts of slime during growth on carbohydrates or lactate, but minor amounts are formed during growth on O₂/H₂ or on alcohols. No correlation between slime formation and oxygen tolerance during nitrogen fixation is ob-

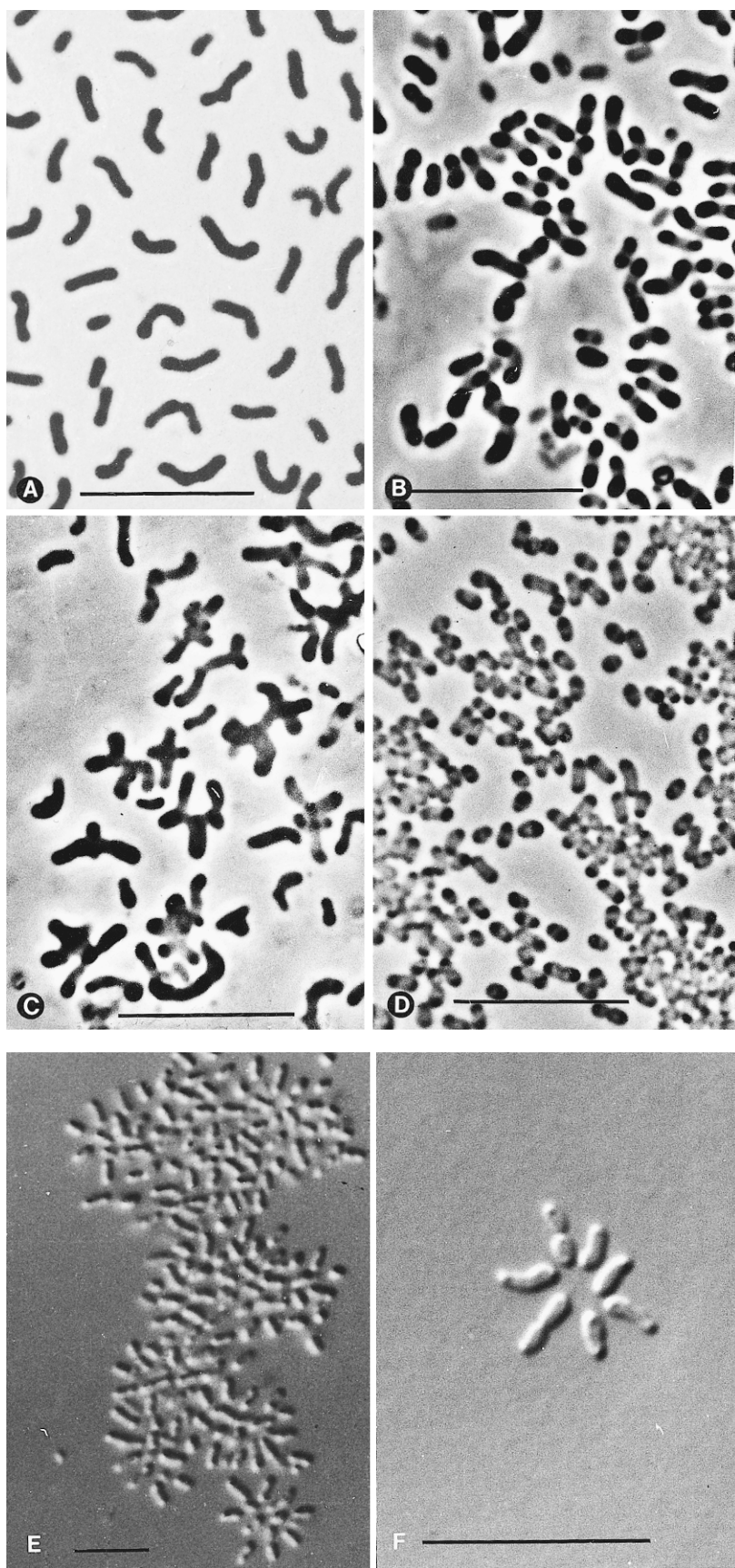


FIGURE BXII.α.241. Cells of *Xanthobacter autotrophicus*. *A*, strain 14g grown on malate in nonagitated liquid culture, showing irregular vegetative cells. *B–D*, strain JW 33 (reference strain). *B*, grown lithoautotrophically under conditions of nitrogen fixation, showing rod-shaped cells and palisade-like formations. *C*, grown in nutrient broth (0.7%) containing 0.1% succinate, showing the typical branched cell formation. *D*, grown on *n*-propanol in the presence of ammonium, showing coccoid cell formation. *E–F*, strain 14g, showing cell aggregation (“star-formation”) on nutrient broth medium. This kind of aggregation is only observed after 2–5 h upon transfer into liquid nutrient broth medium under nonagitated growth conditions. All bars = 10 μm. (Parts *A* and *E* reproduced with permission from K. Schneider et al., *Archives of Microbiology* 93: 179–193, 1973, ©Springer-Verlag, Heidelberg. Parts *B* and *C* reproduced with permission from J. Wiegel and H.G. Schlegel, *Archives of Microbiology* 107: 139–142, 1976, ©Springer-Verlag, Heidelberg. Part *F* reproduced by permission from M. Reh.)

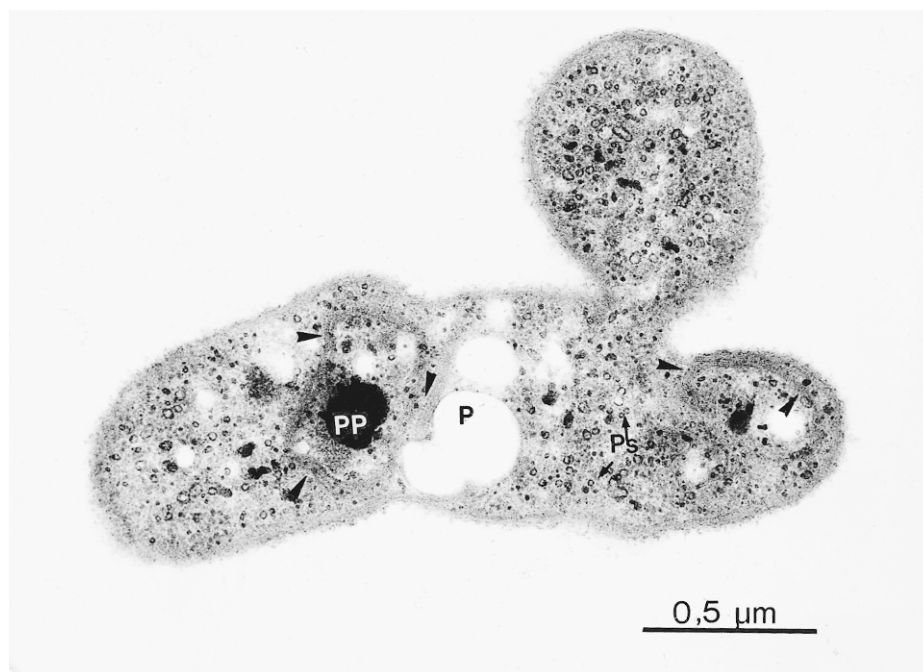


FIGURE BXII.α.242. Ultrathin section of *Xanthobacter autotrophicus* strain 14g grown on a succinate-containing medium. The cells were prepared by glutaraldehyde-osmium tetroxide fixation, uranyl acetate block staining, and lead citrate poststaining; preparation by A. Walther-Mauruschat. *P*, poly-β-hydroxybutyrate; *PP*, polyphosphate; *Ps*, small-type polyphosphate. (Reproduced with permission from J. Wiegel et al., *International Journal of Systematic Bacteriology* 28: 573–581, 1978, ©International Association of Microbiological Societies.)

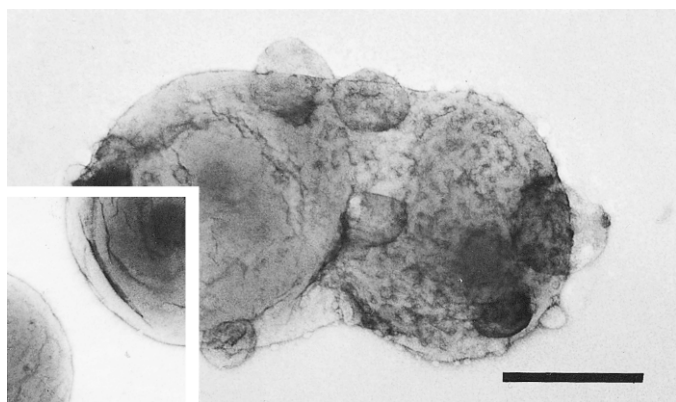


FIGURE BXII.α.243. Demonstration of the presence of lipopolysaccharides in *Xanthobacter autotrophicus*. Negatively stained with 3% uranyl acetate, pH 5. Polymyxin B-treated cell of reference strain JW 33 showing lipopolysaccharide-polymyxin B interactions (bleb formation). The inset shows a control without polymyxin B treatment. Bar = 0.5 μm. (Reproduced with permission from L. Quandt.)

served; however, many of the slime producers grow better than the nonproducers at a high (2–3%) oxygen concentration in the gas atmosphere. Moreover, the majority of the strains produce more slime under N₂-fixing conditions than in the presence of ammonium ions. Beside the motile strains and a very few strains of *X. autotrophicus* that produce traces of slime (e.g., strain JW50), slimeless mutants have been isolated (e.g., mutants of *X. autotrophicus* type strain 7c; Andreesen and Schlegel, 1974), mainly by applying carbon limitation in continuous culture (unpublished results) (Fig. BXII.α.244). The slime becomes soluble at

pH values below 4.5 and above 10.5. With alkaline treatment, the slimy cell aggregates can be separated into single cells as needed for isolation purposes (Wiegel and Schlegel, 1976). The slime is somewhat recalcitrant to microbial degradation under aerobic and anaerobic conditions (Andreesen and Schlegel, 1974), but it exhibits useful drag-reducing properties (e.g., reducing frictions in fluids moving through pipes) (Schubert et al., 1986 and personal communication) and could be used as a viscosifier in oil recovery (Kern, 1985; Wan et al., 1988). A different but interesting property is that *Xanthobacter* slime enhanced the efficiency of gene recombination experiments with *Agrobacterium tumefaciens* (Kawai, 1995).

Polyglutamine capsule All of the 20 strains of *X. autotrophicus* and *X. flavus* tested (other species have not been tested) produce an α-polyglutamine capsule that is located between the cell wall and the slime. This polymer is not separated from the cells during an alkaline (pH 11) treatment to solubilize the slime. The glutamine polymer is not found on cells grown under N₂-fixing or chemolithoautotrophic conditions. In contrast to the slime, this polymer is reutilized in the late stationary growth phase and under N-limitation (J. Wiegel, unpublished results). *Flexithrix* is the only other genus known that produces a similar α-polyglutamine capsule (Kandler et al., 1983).

Storage material *Xanthobacter* species deposit poly-β-hydroxybutyrate (PHB) as a reserve material under heterotrophic as well as under chemolithoautotrophic conditions (Fig. BXII.α.242). PHB formation occurred in all tested strains and was analyzed in more details in strains of *X. autotrophicus*, *X. flavus*, and *X. agilis*. In the stationary growth phase, heterotrophically grown cells contain between 5 and 600 mg PHB/g dry weight of cells, depending on the strain and on the carbon source. Growth on

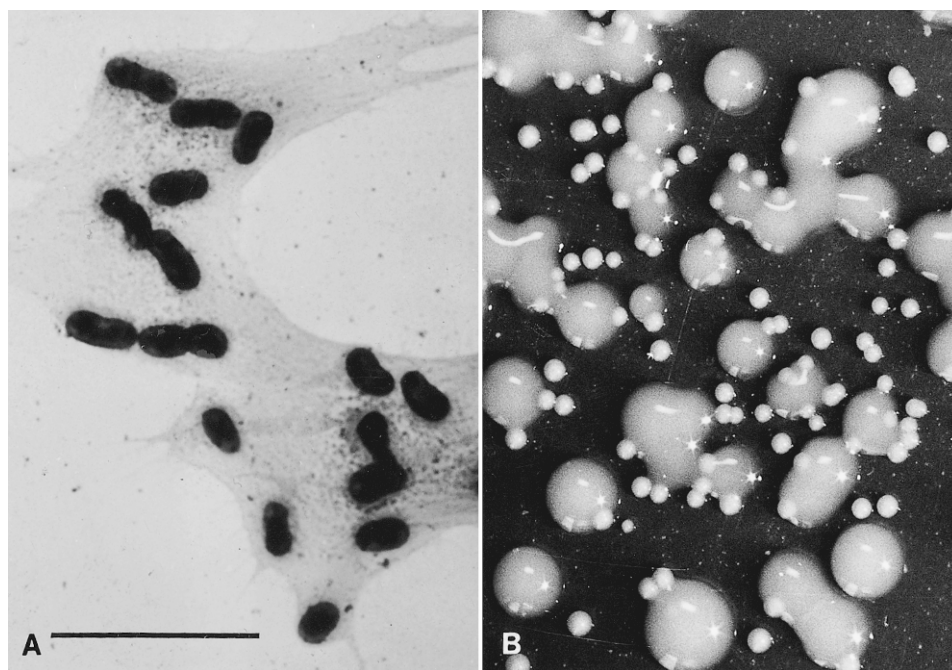


FIGURE BXII.α.244. A, Slime production by *Xanthobacter autotrophicus*. Cells and slime of the type strain (DSM 432) stained negatively by uranyl acetate. Bar = 5 μ m. (Reproduced with permission from N. Tunail and H.G. Schlegel, Archives of Microbiology 100: 341–350, 1974, ©Springer-Verlag, Heidelberg.) B, Colonies from the wild type (large, slimy colonies) and from the slimeless mutant (small, dark yellow colonies) of DSM 432. (Reproduced with permission from M. Andreesen and H.G. Schlegel, Archives of Microbiology 100: 351–361, 1974, ©Springer-Verlag, Heidelberg.)

fructose usually results in a high PHB content, whereas growth on succinate results in very low values (less than 15 mg PHB/g dry weight). For example, reference strain *X. autotrophicus* JW33 has 668, 250, and 8 mg PHB/g dry weight when grown on fructose, sucrose, and succinate, respectively (J. Wiegel, unpublished data), based on quantification by spectroscopy after extraction (Jüttner et al., 1975).

Nitrogen fixation and nitrogenase The *Xanthobacter* species described so far are microaerophilic, heterotrophic, and autotrophic (requiring decreased H_2 and O_2 concentrations) N_2 -fixing bacteria, as described in the original reports of nitrogen fixation in *X. flavus* (as “*Mycobacterium flavum*” 301) (Federov and Kalininskaya, 1961) and in *X. autotrophicus* (as “*Corynebacterium autotrophicum*”) (Gogotov and Schlegel, 1974; Wiegel and Schlegel, 1976). The nitrogen-fixing system and its relationship to oxygen have been studied mainly with *X. autotrophicus* strain GZ29 (Berndt et al., 1976, 1978) and *X. flavus* strain 301 (Biggins and Postgate, 1969, 1971). The composition of the nitrogenase components of heterotrophically grown cells of strain GZ29 and strain 301 is similar to that of other nitrogenases in regard to metal and sulfur content and amino acid composition. As in other aerobic diazotrophs, the nitrogenase system seems to be loosely associated with membranes. The protection of the nitrogen-fixing system in *Xanthobacter* might be due to some conformational protection for the nitrogenase, the respiration of associated aerobic microorganisms in the natural habitat, or the respiratory activity of *Xanthobacter* itself. The latter possibility seems unlikely, since the respiratory rate of *Xanthobacter* is about one to two orders of magnitude lower than that in *Azotobacter* (Biggins and Postgate, 1971). A possible contribution of slime production to protection against oxygen damage to the nitro-

genase could not be demonstrated for *Xanthobacter autotrophicus* (Wiegel, unpublished results). Additional information concerning N_2 -fixation in two more well-characterized strains can be summarized as follows:

In *X. autotrophicus* strain GZ29 (Berndt et al., 1976, 1978), the growth rates with ammonium as the nitrogen source and sucrose as the carbon source are highest at an oxygen partial pressure of 0.15 atm, whereas with N_2 as the sole nitrogen source the maximum growth rates occur at 0.014 atm. In the whole cell assay for acetylene reduction, the optimal oxygen partial pressure is 0.0036 atm. Even in the absence of any detectable oxygen, acetylene is reduced linearly for more than 1 h. The acetylene reduction activity has a very narrow pH optimum at 6.8. The nitrogenase is not cold labile. The overall efficiency of nitrogen fixation is 22 mg nitrogen fixed/g of sucrose consumed, but in the early exponential growth phase, values up to 65 mg/g have been found.

In *X. flavus* strain 301 (Biggins and Postgate, 1969, 1971) heterotrophic growth in the presence of ammonium is not dependent on the O_2 partial pressure in the range of 0.01–0.20 atm, but with N_2 as the sole nitrogen source, growth and nitrogen fixation have an optimum at 0.1 atm N_2 . The optimal pO_2 for acetylene reduction by whole cells is 0.05 atm. The nitrogenase activity of cell-free extracts stirred under 0.2 atm O_2 decays exponentially, with a half-life of about 5 min. ATP enhances the sensitivity to O_2 , presumably because of the formation of an active enzyme conformation. As with nitrogenases from other sources, the enzyme reduces H^+ to H_2 , KCN to CH_4 , and CH_3NC to CH_4 , C_2H_4 and C_2H_6 . The optimal concentration of ATP in the cell-free extract for acetylene reduction is 4–8 mM. As in other aerobic diazotrophs, but in contrast to the systems of *Clostridium*

pasteurianum, pyruvate fails to promote acetylene reduction by cell-free extracts.

Ammonium transport Wiegel and Kleiner (1982) postulated that *Xanthobacter* possesses an active ammonium (methyl ammonium) transport system similar to that of other diazotrophs. Methyl ammonium can serve as a sole carbon and nitrogen source (unpublished data). S. Kustu (personal communication) disputes this interpretation and maintains that there is no active ammonium transport system in any bacterium.

Hydrogenase The strains of *X. autotrophicus* studied (e.g., strains 14g, 7c, GZ29) contain a membrane-bound uptake hydrogenase. Like the majority of membrane-bound hydrogenases, this enzyme is not able to reduce NAD directly; instead, it channels electrons into the electron transport chain. A soluble, NAD-reducing hydrogenase is not present (Schneider and Schlegel, 1977). In strain 7c the enzyme is constitutive, and cells grown on various organic substrates and ammonium contain 10–100% of the hydrogenase activity compared to chemolithoautotrophically grown cells (Tunail and Schlegel, 1974). In strain 14g the hydrogenase is a strictly inducible enzyme (Schneider et al., 1973). In strain GZ29 the hydrogenase is apparently a constitutive enzyme; its specific activity (up to 31 mol H₂/h/mg protein in N₂-fixing cells) depends on the oxygen concentration during growth (Berndt and Wölfe, 1979; Pinkwart et al., 1979). Although the enzyme has been solubilized from the membranes and partially purified (Schink and Schlegel, 1980), a detailed description of it is still lacking. Immunological comparison of the membrane-bound hydrogenase of *Alcaligenes eutrophus* strain H16 reveals no relationship to that of *X. autotrophicus* GZ29 (Schink and Schlegel, 1980). Furthermore, an antiserum against the hydrogenase of *X. autotrophicus* strain GZ29 did not react with the membrane extracts of strains 7c, 14g, and 12/60/x. This indicates major differences between the strains. The hydrogenase of the motile strain MA2 is apparently a loosely membrane-bound enzyme that tends to form aggregates and exhibits an unusual high specific activity (Pinkwart et al., 1979, 1982).

Growth conditions The spectrum of carbohydrates utilized by about 80 tested strains of *X. autotrophicus* is normally limited to fructose and/or sucrose and/or—in the presence of traces of yeast extract—mannose. *X. tagetidis* can also grow on galactose and lactose as the sole carbon and energy source. Some strains (*X. autotrophicus* strain 14g and *X. agilis* strains MA2 and SA35 (Jenni and Aragno, 1987)) do not utilize carbohydrates at all, whereas Malik and Claus (1979) reported that strain *X. autotrophicus* DSM 685 could use a wider than normal range of carbohydrates. However, no 16S rRNA sequence or DNA–DNA hybridization data exist on these strains for a definite placement in the proposed species. Some strains of *X. flavus* can utilize a few more sugars, including glucose. Normally *X. autotrophicus*, *X. agilis*, and *X. tagetidis* cannot use glucose, although the pertinent catabolic enzymes are induced during growth on fructose (Opitz, 1977; Padden et al., 1997). However, glucose-utilizing *X. autotrophicus* and *X. flavus* (others not tested) cells appear spontaneously when cultures are incubated for a prolonged time (2–3 weeks) in the presence of glucose. These adapted strains (transport mutants) grow very well on glucose; however, they lose this ability again after growing on other carbohydrates for one or two generations (J. Wiegel, unpublished data). Using the API 20E test, *X. tagetidis* was positive for acetoin production from glucose.

Catabolite repression is exerted by H₂ on the utilization of organic substrates; however, the extent of this effect varies with

the organic substrate and the strains studied. Some strains need CO₂/CO₃²⁻ for induction of the enzymes for heterotrophic growth after transition from lithotrophic to organotrophic conditions (Schneider et al., 1973).

The doubling times reported for various strains vary between 1.5 and 5.0 h (heterotrophic conditions) and 3.0 to over 12.0 h (chemolithoautotrophic conditions). Under N₂-fixing conditions, the doubling time is slightly longer (Wiegel and Schlegel, 1976; Berndt et al., 1978).

Traces of yeast extract shorten the lag phase after transition from heterotrophic to lithotrophic conditions. Many strains of *X. flavus* and three strains of *X. autotrophicus* are reported to require biotin (Aragno, 1975). High cell yields during mass culture under autotrophic conditions are obtained when oxygen levels are raised to match the increasing growth. For example, for autotrophic growth with ammonium as the nitrogen source, an O₂ level of 5–8% (v/v) is usually optimum; at optical densities higher than 1.0 (at 600 nm), 10–15% O₂ is sufficient, and at optical densities higher than 2.5 (at 600 nm), 20% O₂ in the gas atmosphere is required for good growth. The oxygen-resistant strain, *X. autotrophicus* Y38, requires nickel for optimal growth (Nakamura et al., 1985).

For strain *X. autotrophicus* GZ29, intact cells are able to reduce acetylene under anaerobic assay conditions, indicating the presence of a fermentative energy regenerating system. So far, however, growth has not been observed under anaerobic conditions and no strict anaerobic metabolic activity has been ascertained (Berndt et al., 1976; Arzumanyan et al., 1997). The finding of a typical methanogenic cofactor, coenzyme M, in *Xanthobacter*, suggests that this issue needs to be revisited, although the proposed role of the cofactor is in the carboxylation of aliphatic epoxide in the aerobic alkene utilization pathway (Allen et al., 1999; Krum and Ensign, 2000; Sauer and Thauer, 2000).

The typical pH for growth is between 6.5 and 8.0, with the optimum around 7.5 for most strains. Although usually no acids are formed from carbohydrates or alcohols, the formation of acidic slime and the consumption of ammonium ions cause the pH of the medium to decrease. This decrease has to be counterbalanced by adding alkali to maintain optimal growth. During growth on organic acids, the pH shift is small, but depending on the slime production of the strain used, it can be in either the acidic or alkaline direction.

Antibiotics Tested strains of *Xanthobacter autotrophicus* and *X. flavus* are sensitive to the following antibiotics at 100 µg/ml of growth medium: penicillin (MIC ~1 µg/ml), novobiocin, and polymyxin B. They are resistant to erythromycin and bacitracin at 200 µg. Violet red-bile medium (Oxoid), deoxycholate medium (Oxoid), and tellurite agar (selective for coryneform bacteria) support growth of the majority of the isolated strains. Both *Xanthobacter* species grow on mineral medium supplemented with an appropriate carbon source and 10⁻⁵ M crystal violet. However, the colonies formed are red instead of the blue color that is characteristic of other Gram-negative bacteria (Wiegel et al., 1978).

In contrast, *X. tagetidis* is resistant to 1 U penicillin and 5 µg/ml tetracycline but sensitive to 25 µg/ml of ampicillin and 30 µg/ml of chloramphenicol (Padden et al., 1997).

Metabolic pathways Carbohydrates and gluconate are degraded via the Entner–Doudoroff pathway, as has been shown for *X. autotrophicus* strains by determining enzyme activities after growth on various substrates (Tunail and Schlegel, 1972, 1974;

Schneider et al., 1973). In addition, radiorespirometric studies indicate that the pentose phosphate pathway is concomitantly used to a significant degree (Opitz and Schlegel, 1978). *X. autotrophicus* Py2 utilizes an ATP-utilizing acetone carboxylase when growing on acetone (Sluis et al., 1996).

C₁-carbon utilization CO₂ is mainly fixed via the ribulose biphosphate pathway (Bowien and Schlegel, 1981; Lehmick and Lidstrom, 1985; Meijer et al., 1991; Meijer 1994; Shively et al., 1998). The key enzyme, ribulose biphosphate carboxylase, is inducible. In addition, phosphoenolpyruvate carboxylase activity occurs. Radiorespirometric experiments with ¹⁴CO₂ or ¹⁴CH₃OH as substrates yielded radioactive malate. Other experiments suggest that methanol is presumably utilized via CO₂ and the ribulose biphosphate cycle (Opitz, 1977). Methanol is oxidized to CO₂ via a methanol dehydrogenase, which is of the normal type (mol. wt. of about 120,000) stimulated by NH₄⁺ and contains 2 mol of the coenzyme pyrroloquinoline quinone (PQQ) (J. A. Duine, personal communication). The glucose-6-phosphate dehydrogenase is allosterically inhibited by phosphoenolpyruvate (PEP) (Tunail and Schlegel, 1972; Opitz and Schlegel, 1978). The triose phosphate isomerase is the same enzyme that is formed during chemolithoautotrophic and heterotrophic growth (Meijer et al., 1997).

Carbon monoxide is not oxidized to CO₂ by *X. autotrophicus* or *X. flavus* strains (others not tested) and cannot serve as an energy or carbon source for lithotrophic growth (O. Meyer, personal communication). Thiosulfate can substitute for H₂ as a substrate for lithotrophic growth and provide energy for CO₂-fixation (Friedrich and Mitrenga, 1981; J. Wiegel, unpublished data, six strains of *X. autotrophicus* tested).

Alkene utilization and epoxide formation The use of various alkenes such as ethene, propene, butene, and 1,3-butadiene through oxidation by monooxygenases has been studied in detail on the physiological and biochemical level mainly in *X. autotrophicus* strain Py2. The oxidation leads to the formation of epoxides, which then are further metabolized by epoxide hydrolases, and carboxylases (Swaving and deBont, 1998, and older literature cited therein). The use of *Xanthobacter* strains for the commercial removal of propene from waste gas has been proposed (Reij and Hartmans, 1996).

Chloroalkenes and haloaromatic compounds Much work has been done on the haloalkene and haloaromatic compounds degradation by *Xanthobacter* strains, mainly with *X. autotrophicus* GJ10 and on chlorobenzenes with *X. flavus* 14p1 (Spiess et al., 1995; Spiess and Gorisch, 1996; Sommer and Gorisch, 1997). A novel dehalogenase has been found and purified and the crystal structure elucidated. Strain GJ10 contains two dehalogenating enzymes one for haloalkenes and the other for halogenated carboxylic acids (Prince, 1994). Strain *X. autotrophicus* Py2 has a novel pathway for degradation of epichlorohydrin. This strain also degrades trichloroethylene during growth on propene (Reij et al., 1995) as does *X. autotrophicus* GJ10 (Inguva and Shreve, 1999).

Genetics Intraspecific gene transfer has been detected in crosses involving the strains GZ29, GZ27, and JW50 of *X. autotrophicus*, which produce only traces of slime. Strain GZ29 was used to study the transfer more closely. The involvement of a defective generalized transducing bacteriophage as well as conjugational gene transfer have been described (Wilke and Schlegel, 1979; Wilke, 1980). A plasmid has also been identified in strain GJ10 (Bergeron et al., 1998). The bacteriophage CA3 was

detectable only by its transducing activity and by electron microscopy; it did not form plaques. The genetic markers (resistance, auxotrophy, pigmentation) were transducible at frequencies of about 10% per marker and per phage particle. No cotransduction of markers was detected. Mating experiments on agar plates have revealed a recombination system requiring direct cell contact. It allowed the transfer of large chromosomal segments at low frequency. All partners used functioned as donors as well as recipients. Two groups of closely linked markers have been found (Wilke, 1980). In addition, an electrotransformation system that is also applicable to other strains has been developed for *X. autotrophicus* GJ10. Transformation rates up to 2×10^6 cells transformed per µg DNA were obtained (Swaving et al., 1996; Swaving and deBont, 1998). A triparental mating system was used to express the dehalogenase in other *Xanthobacter* strains including type strain 7c (Wilke, 1980; Janssen et al., 1989). For *X. autotrophicus* strain Py2, mutants defective in epoxyalkane degradation were complemented with DNA fragments (Swaving et al., 1995).

Cytochromes, ubiquinones, and fatty acids *X. autotrophicus* (only strain 14g has been investigated) contains cytochromes *a*, *b* (two different ones), *c*, and *o*, irrespective of the growth conditions. The amounts of cytochromes *a*, *b*, and *c* type were 0.03, 0.4–0.52, and 0.36 µmol/g of particle protein, respectively (Bernard et al., 1974). Ubiquinones Q-10 (major), Q-9, and Q-8 are present in *X. autotrophicus* and *X. flavus* (Collins and Jones, 1981; Urakami et al., 1995a, and unpublished data). The cellular fatty acids are high in C_{18:1} and include C_{18:0}, C_{16:0} 3OH, and, in *X. agilis*, cyclo C_{19:0} cyclo ω7c (Urakami et al., 1995a; Wiegel, unpublished results) (Table BXII.α.189). Menaquinones have not been found in these species (Wiegel et al., 1978). *X. autotrophicus* and *X. flavus* contain the coenzyme pyrroloquinoline quinone after growth on methanol (J.A. Duine, personal communication).

Ferredoxin *Xanthobacter flavus* (previously called "*Mycobacterium flavum*" 301) contains a [4Fe-4S]₂ and a [4Fe-4S]-ferredoxin. It is likely that the latter is a [3Fe-3S]-ferredoxin. *X. autotrophicus* GZ29 contains two different [4Fe-4S]₂-ferredoxins (Bothe and Yates, 1976; Berndt et al., 1978; Yates et al., 1978; M.G. Yates, personal communication). The ferredoxin of *X. autotrophicus* GZ29 exhibits EPR features in the oxidized as well as in the reduced state, which is in contrast to the ferredoxins of *Azotobacter vinelandii* and *X. flavus*. Thus, it is possible that *X. autotrophicus*, at least strain GZ29, contains ferredoxins unique among N₂-fixing bacteria. Additional strains need to be analyzed before conclusions regarding generic diversity can be drawn. There is no evidence for the presence of constitutive flavodoxins in *X. flavus* 301 (Bothe and Yates, 1976) or in *X. autotrophicus* GZ29. In the latter strain, the ferredoxins probably serve as direct electron donors for the nitrogenase (Schrautemeier, 1981).

Habitats The type strain of *X. autotrophicus*, strain 7c, was isolated from black mud of a pond near Göttingen during enrichments for propane-oxidizing bacteria (Siebert, 1969). However, strain 7c and all other strains tested do not utilize propane. Typical strains of *X. autotrophicus* and *X. flavus* can be specifically isolated from wet soil and mud containing organic material including wetland rice fields (Oyaizu-Masuchi and Komagata, 1988; Reding et al., 1991). A highly specific isolation procedure is based on the ability to fix nitrogen under chemolithoautotrophic growth conditions and specific colony morphology and color on succinate-containing nutrient broth plates after an alkaline treatment (Wiegel and Schlegel, 1976). Yellow colonies containing

TABLE BXII.α.189. Fatty acid analysis of *Xanthobacter* strains^a

Fatty acid	<i>X. autotrophicus</i> 7c	<i>X. autotrophicus</i> 7cSF	<i>X. agilis</i> SA35	<i>X. flavus</i> 301	<i>X. flavus</i> 4-14H	<i>X. flavus</i> JW-KR1	<i>X. flavus</i> W-KR2
C _{16:0}	4.83	3.48	10.32	2.67	3.84	2.68	1.19
C _{16:0} 3OH	1.32	0.98	0.74	0.73	1.45	1.69	1.45
C _{16:1} ω7c	5.04	6.24	3.45	0	1.86	0.52	0
C _{17:0}	0	0	0	0	0	0	0.76
C _{17:0} cyclo	2.45	1.23	0	0	0	0	0
C _{18:0}	1.98	1.53	3.25	2.65	1.19	1.79	1.99
C _{18:1} ^b	76.81	81.64	57.44	91.9	91.66	93.31	93.01
C _{18:2} ω6,9c and C _{18:0}	0	0	1.13	0	0	0	0
C _{19:0} cyclo ω7c	5.99	3.02	19.68	1.84	0	0	0.86
C _{19:1} ω12t	0	0	1.4	0	0	0	0
C _{20:1} ω9t	1.55	1.9	1.35	0	0	0	0.74

^aData from Reding (1991). Samples were prepared and analyzed by Microbial ID Inc. (Newark, DE).

^bValue given is the sum of C_{18:1} ω7c, C_{18:1} ω9t, and C_{18:1} ω12t.

branched cells (Fig. BXII.α.241) are probably strains of *Xanthobacter*. Thus, it appears that *Xanthobacter* is ubiquitous in sediment and wet soil samples—including from wet rice fields and marine mud samples—containing organic material.

Strains from *X. autotrophicus* and *X. flavus* can be isolated as associative nitrogen-fixing microaerophiles from rice roots (Reding et al., 1991) and grass roots (Wiegel, unpublished data). *X. taetidis* was isolated from soil around the roots and roots of the marigold plant (*Tagetes*), suggesting that it is also an associative nitrogen-fixing species (Padden et al., 1997). *X. agilis* has been isolated from the water column of a Swiss lake (Aragno, 1975). Roots of coconut palms (*Cocos nucifera*) contained 10³ cells per g dry weight (R. Prabhu, personal communication). One *X. flavus* strain originated from a marine sediment sample (Lidstrom-O'Connor et al., 1983; Meijer et al., 1990). *X. autotrophicus* and *X. flavus* isolates have also been found as members of a wood degrading community in wooden pipelines (2-m diameter) from hydroelectric power plants (Line, 1997). Another unusual environment in which *Xanthobacter* has been identified by 16S rDNA assay is in the accessory nidamental glands (ANG) of female cuttlefish and in the myopsid squids, where it occurs as a symbiont (Grigioni et al., 2000).

Pathogenicity No clinical reports of infections of humans or animals by *Xanthobacter* species have been published. In various laboratory settings, however, pathological effects have been observed. This includes the killing of embryos in normally pregnant mice by intraperitoneal injection of 2 × 10⁸ cells of *X. autotrophicus* per g body weight (Manna and Sadhukhan, 1991). In addition, *X. flavus* has exhibited cytological effects and chromosomal aberrations in mouse and human cell cultures (Manna and Sadhukhan, 1992, 1993). No plant pathogenic effects have been reported.

MAINTENANCE PROCEDURES

All strains of *Xanthobacter* can be readily maintained autotrophically on minimal media containing vitamins and 0.02% yeast extract. Cultures grown chemolithoautotrophically can be kept at 2–5°C in liquid medium for about 10 months and in sealed agar slants for more than 15 months; in the presence of 60% (v/v) glycerol, they can be stored for at least 3 years at –20°C. Cultures in liquid nutrient broth or on nutrient agar plates tend to lose viability within 30 d at 4–25°C. For long-term preservation, the cultures are lyophilized in the presence of skim milk with 10% (wt/wt) honey; they have been kept in this form for more than 20 years without significant loss of viability (Malik, 1975, personal communication).

DIFFERENTIATION OF THE GENUS *XANTHOBACTER* FROM OTHER GENERA

Originally two main phenotypic characters were considered to be definitive for *Xanthobacter*: (a) nitrogen fixation under heterotrophic conditions as well as under chemolithoautotrophic conditions (with H₂ serving as the electron donor for respiration), and (b) the presence of the egg-yolk yellow color due to zeaxanthin dirhamnoside. However, nitrogen fixation with H₂ utilization also occurs in several other diazotrophic bacteria (e.g., *Rhizobium*, *Azotobacter*, and *Azospirillum*). Thus, besides determining the relatedness of new isolates to *Xanthobacter* based on the comparison of 16S rDNA sequences, the following special characteristics should be tested before strains are assigned to the genus *Xanthobacter*:

Chemolithoautotrophic growth on H₂ + CO₂ + O₂.

Microaerophilic N₂-fixation.

Pleomorphic cells when grown in nutrient broth containing TCA cycle intermediates.

The presence of zeaxanthin dirhamnoside (tested as described by Wiegel (1986)).

Since the Gram reaction (even with the modified procedures) is often doubtful with these species, other methods such as the polymyxin B-lipopolysaccharide interaction (Wiegel and Mayer, 1978; Wiegel and Quandt, 1982), Fig. BXII.α.243 should be used to determine the Gram-type (Wiegel, 1981).

The mol% G + C of the DNA should be in the range 65–70.

See phylogenetic comments below for a differentiation from the closely related (based on 16S rDNA sequence analysis) *Aquabacter* and *Azospirillum*.

Xanthobacter strains were originally described as *Mycobacterium* and as *Corynebacterium* (coryneform bacteria) because of their pleomorphic cell shape, their tendency to exhibit a positive Gram staining reaction, their high mol% G + C value, and the impression of a “snapping-type” or “palisade” cell formation. However, in contrast to *Arthrobacter* and coryneform bacteria, snapping occurs because of the presence of adhesive slime and not because of the rupture of connective cell walls (Figs. BXII.α.241 and BXII.α.244). Moreover, *Xanthobacter* is a Gram-type negative organism (Wiegel, 1981). The main carotenoid, zeaxanthin dirhamnoside, separates recognized *Xanthobacter* from other yellow-pigmented chemolithotrophic or N₂-fixing bacteria of other genera. Zeaxanthin has also been found in some strains of *Flavobacterium* that have a high mol% G + C value; however, members of this group neither fix N₂ nor grow chemolithoautotrophically, and do not show pleomorphism similar to that of *Xanthobacter*.

(Oyaizu-Masuchi and Komagata, 1988). The high mol% G + C of *Xanthobacter*, its pleomorphism (on succinate–nutrient broth media), and the ability to utilize short aliphatic alcohols are further properties that separate this genus from many other hydrogen-oxidizing bacteria.

TAXONOMIC COMMENTS

Xanthobacter belongs to the *Alpha*proteobacteria (Rainey and Wiegand, 1996), in agreement with its previous assignment to the fourth RNA superfamily of the Gram-type negative bacteria (De Smedt et al., 1980). Before 16S rDNA sequence analysis was completed, the genus *Xanthobacter* was regarded as a well-defined genus with easily recognizable traits (see above). However, 16S rDNA sequence analysis places two other organisms—*Azorhizobium caulinodans* (Dreyfus et al., 1988) and *Aquabacter spiritensis* (Irgens et al., 1991)—among the species of *Xanthobacter*, thereby converting the well-defined genus to a cluster—the *Xanthobacter*–*Azorhizobium*–*Aquabacter* cluster. The placement of *Azorhizobium* is in agreement with a previously published rRNA cistron similarity dendrogram (De Ley, 1992). *Azorhizobium caulinodans* differs from all *Xanthobacter* species by its absence of the characteristic yellow color, failure to grow on carbohydrates, methanol, or methylamine, and by its inability to fix CO₂; moreover, unlike *Xanthobacter*, it shows nodulation of plant stems. *Aquabacter spiritensis* (Irgens et al., 1991) differs from *Xanthobacter* by containing gas vacuoles, showing no sugar or methanol utilization, failing to grow under autotrophic conditions, and by an absence of pleomorphic cell shapes. For both *Aquabacter* and *Azorhizobium*, zeaxanthin dirhamnoside or a precursor thereof has not been demonstrated, although the organisms probably have not been care-

fully analyzed for its presence. Since *Aquabacter* and *Azorhizobium* apparently do not share several of the key properties used to define the genus *Xanthobacter*, it is presently not quite clear how to solve the problem of their taxonomy. To make a better decision on what defines this phylogenetic group beside its relatedness on the 16S rDNA sequence level, the author suggests waiting until more novel and unusual strains and 16S rDNA sequences have been described. One possibility is that, based on the 16S rDNA sequence data, all isolates including the strains presently named *Azorhizobium* or *Aquabacter* and similar strains might be combined as species in an emended genus *Xanthobacter*, since this genus name would have seniority. Another possibility is that *X. agilis*, *X. flavus*, and *X. tagetidis* might be transferred to new genera. At present, an assignment of novel strains to the genus *Xanthobacter*, *Aquabacter*, and *Azorhizobium* should definitely include 16S rDNA sequence analysis and if possible DNA–DNA hybridization data to aid in solving this question.

Two not validly published *Blastobacter* species were reassigned to two novel *Xanthobacter* species *X. viscosus* 7d and *X. aminoxidans* 14d (Doronina and Trotsenko, 2003). Both strains were originally isolated from activated sludge and exhibited yellow branching cells and other typical characteristics of *Xanthobacter* species. However, cells reproduced by budding, thus requiring an emendation of the *Xanthobacter* genus description to include this feature. Closest neighbors in the 16S rDNA based phylogenetic tree are *X. autotrophicus* and *X. flavus*, respectively. The creation of *X. aminoxidans* based on DNA–DNA hybridization data requires a re-evaluation of the *X. flavus* strains including H4-14 with respect to the type strain *X. flavus* 301, since the differences in the 16S rDNA values of the strains are small.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *XANTHOBACTER*

Characteristics useful for the differentiation of the four species currently recognized in the genus *Xanthobacter* are listed in Table BXII.α.190. Because the designated type strain of the type species *X. autotrophicus* strain 7c is a relatively atypical strain, the reference strain *X. autotrophicus* JW33 (DSM 1618) should be used for

comparative studies (Wiegand et al., 1978). However, alkane oxidation and dehalogenation capabilities and thiophene carboxylic acid utilization have not been studied for either of these two strains.

TABLE BXII.α.190. Differential characteristics of the species of the genus *Xanthobacter*^a

Characteristic	<i>X. autotrophicus</i>	<i>X. agilis</i>	<i>X. flavus</i>	<i>X. tagetidis</i>
Cell morphology: pointed ends, twisted	–	–	–	+
Highly pleomorphic on nutrient broth agar + succinate	+	–	+	+
Motility under autotrophic growth conditions	–	+	–	+
Vitamins required for growth (biotin, riboflavin)	d	–	+	–
Sensitivity to chloramphenicol	–	+	–	+
Autotrophic growth at 37°C	+	–	+	+
Growth on nutrient broth	+	–	+	+
Growth on glutamine as carbon source	+	–	–	nd
Growth on citrate	+	–	d	+

^aFor symbols, see standard definitions; nd, not determined.

List of species of the genus Xanthobacter

1. **Xanthobacter autotrophicus** (Baumgarten, Reh and Schlegel 1974) Wiegel, Wilke, Baumgarten, Opitz and Schlegel 1978, 580^{AL} ("Corynebacterium autotrophicum" Baumgarten, Reh and Schlegel 1974, 214.)

au.to.tro'phi.cus. Gr. pref. *auto* self; Gr. n. *trophos* one who feeds; M.L. masc. adj. *autotrophicus* self feeding, referring to the ability of the organism to use CO₂ as a sole carbon source.

The characteristics are as described for the genus and as listed in Tables BXII.α.190 and BXII.α.191. The morphology is depicted in Fig. BXII.α.241.

Habitat: soil, mud, and water. Widely distributed in nature.

The mol% G + C of the DNA is: 69–70 (*T_m*) (Wiegel et al., 1978); 65–68 (*T_m*) (De Smedt et al., 1980); 66–68 (Bd) (M. Aragno, unpublished data).

Type strain: 7c, ATCC 35674, CIP 105432, DSM 432.

GenBank accession number (16S rRNA): X94201.

Additional Remarks: The type strain is atypical of the species, and reference strain DSM 1618 (strain JW33; Wiegel and Schlegel, 1976; Wiegel et al., 1978) should be used for comparative purposes. Reference strains T101 and T102 (ATCC7000551/2) degrade toluene and were isolated from a drainage ditch (Wilmington MA, USA). Strain GJ10 (DSM 3874; ATCC 43050) degrades haloalkanes including dichloromethane (Janssen et al., 1985). Other well-characterized strains can be obtained from the authors who described them.

2. **Xanthobacter agilis** Jenni and Aragno 1988, 136^{VP} (Effective publication: Jenni and Aragno 1987, 257.)

a'gi.lis. L. adj. *agilis* quick, agile, indicating motility.

The general characteristics are as described for the genus and as listed in Tables BXII.α.190 and BXII.α.191. The morphology of *X. agilis* differs somewhat from that of *X. autotrophicus*, *X. flavus*, and *X. tagetidis* in that the branched-cell morphology on succinate-NB medium is much less pronounced and can be observed only after prolonged incubation.

At the time of its description, *Xanthobacter* species were described as nonmotile; this description was changed later (Reding et al., 1992; Reding and Wiegel, 1993). However, *X. agilis* strains are motile under nearly all growth conditions and exhibit a faster movement than that of other species.

Several strains were isolated from a freshwater lake in Switzerland (Jenni and Aragno, 1987). The type strain was isolated from the water column of a small lake in Neuchatel (Switzerland).

The mol% G + C of the DNA is: 68–69 (*T_m*).

Type strain: SA35, ATCC 43847, DSM 338, LMG 16336.

GenBank accession number (16S rRNA): X94198.

3. **Xanthobacter flavus** Malik and Claus 1979, 286^{AL} *fla'vus*. L. adj. *flavus* yellow.

The characteristics are as described for the genus and as listed in Tables BXII.α.190 and BXII.α.191. The properties of *X. flavus* are somewhat similar to those of *X. autotrophicus*, except the nitrogenase system of *X. flavus* 301 differs considerably from *X. autotrophicus* strain GZ29 (Berndt et al., 1978) and all *X. flavus* used to require the

addition of biotin to the culture medium. However, non-biotin-requiring strains have been isolated more recently and identified as *X. flavus* by 16S rDNA sequence analysis. The DNA–DNA hybridization values (*T_m*) between the type strains of *X. flavus* and *X. autotrophicus* is about 25%. Furthermore, in comparison with *X. autotrophicus*, the type strain 301 exhibits a higher sensitivity to oxygen under autotrophic conditions. The range of carbohydrates that can be used is no longer regarded as a valuable property for differentiating these two species as previously proposed (Malik and Claus, 1979).

Isolated from turf podzol soil in the U.S.S.R, rice paddy sediments, marine sediments, sewage in the Netherlands, and wetland rice roots.

The mol% G + C of the DNA is: 68–69 (*T_m*).

Type strain: 301, ATCC 35867, DSM 3770; IFO 14759, NCIB 10071.

GenBank accession number (16S rRNA): X94199.

Additional Remarks: Reference strains include rice root isolate JW-KR2 (ATCC 51492); strain 14p1 (DSM 10330), which degrades 1,4-dichlorobenzene and was isolated from contaminated soil by Sommer and Gorisch (1997); and strain H4-14 of Meijer et al. (1990). The type strain, 301, was misclassified by Federov and Kalininskaya (1961) as "*Mycobacterium flavum*" strain 301.

4. **Xanthobacter tagetidis** Padden, Rainey, Kelly and Wood 1997, 400^{VP}

ta.ge.ti'dis. M.L. n. *tagetidis* of *Tagetes* the marigold genus of flowering plants.

The main characteristics are as described for the genus and as listed in Tables BXII.α.190 and BXII.α.191. The main properties of *X. tagetidis* are similar to those of the other species in respect to the characteristic yellow color of zeaxanthin dirhamnoside, pleomorphism on succinate-NB medium, production of copious amounts of slime while growing on carbohydrates, and restricted utilization of carbohydrates. Although they grow poorly, the wild type strains are able to grow more readily on glucose than most other strains tested belonging to the other three species. It can be specifically isolated from soil around the roots of marigold plants (*Tagetes patula* and *T. erecta*). *X. tagetidis* is so far the only *Xanthobacter* species that can utilize substituted thiophenes. Whether or not the ability to grow on substituted thiophenes is restricted only to *X. tagetidis* (Padden et al., 1997, 1998) or also can be found among strains belonging to the other species is presently unknown. Similar to *X. agilis*, *X. tagetidis* is motile under most growth conditions.

To date, two strains were described that were isolated from sludge (strain DSM 11602) and from soil around marigold plants (strain TagT2C), respectively, in England. The type strain was isolated from composted root balls of *T. patula*.

The mol% G + C of the DNA is: 68–69 (*T_m*).

Type strain: TagT2C, ATCC 700314, DSM 11105.

GenBank accession number (16S rRNA): X99469.

Additional Remarks: Several strains with properties other than described above have been isolated and are under investigation. However, several of them, although biochemically well studied, have not been deposited in a culture collection or assigned a species name at this time.

TABLE BXII.α.191. Other characteristics of the species of the genus *Xanthobacter*^a

Characteristic	<i>X. autotrophicus</i>	<i>X. agilis</i>	<i>X. flavus</i>	<i>X. taetidis</i>
Cell diameter, µm	0.4–0.8	0.7	0.5–0.7	0.5
Cell length, µm	0.8–4.0 ^b	1.1–3.6	1.0–2.5 ^c	1
Motility	+ ^d (on propanol)	+	+ ^d (on propanol)	+
Water-insoluble zeaxanthin pigment produced ^d	+	+ (low conc.)	+	+
Slime produced ^c	+	Traces	+	+
α-Polyglutamine capsule-like material produced	+	nd	+	nd
Polyphosphate granules formed	+	+	+	+
Intracellular poly-β-hydroxybutyrate formed	+	+	+	nd
<i>Growth at:</i>				
15°C	Weak	+	Weak	+
28–32°C	+	+	+	+
37°C	Weak	–	Weak	+
45°C	d and weak	–	–	–
pH range for growth	5.0–9.0	nd	nd	6.8–8.7 (opt. 7.6–7.8)
<i>Growth in the presence of:</i>				
25% (w/v) NaCl	d	nd	+	nd
5.0% (w/v) NaCl	d	–	–	nd
7.5% (w/v) NaCl	–	–	–	nd
1.0% (w/v) dodecylsulfate	–	nd	–	nd
10 uM crystal violet	d	nd	+	nd
Growth on tellurite agar	d	nd	+	nd
Growth on H ₂ (with O ₂) as energy source	+	+	+	+
Growth on thiosulfate as energy source	+	+	+	+
Utilization of CO ₂ as sole carbon source	+	+	+	+
Growth in submerged liquid culture	+	+	+	+
Strictly respiratory type of metabolism	+	+	+	+
Nitrate as terminal electron acceptor	–	–	–	–
Heterotrophic growth under air atmosphere	+	+	+	+
<i>Autotrophic growth occurs:</i>				
Up to 20% O ₂ atmosphere	+	+	– (d)	+
Only below 5% O ₂ atmosphere	(<10% air)	–	+	–
<i>Nitrogen fixation:</i>				
Microaerobic conditions	+	+	+	+
Air (100%)	–	–	–	+
Growth requirement: Biotin	–	–	(+)	–
Membrane-bound hydrogenase (uptake) that does not reduce NAD	+	+	+	nd
<i>Hydrogenase activity:</i>				
Inducible only	–	nd	+	nd
Inducible or constitutive	+	nd	–	nd
Oxygen-labile	+	nd	–	nd
Carbohydrates catabolized via the Entner–Doudoroff and pentose phosphate pathways	+	–	+	nd
<i>Sole carbon source:</i>				
Formate, acetate, propionate, butyrate, pyruvate, succinate, fumarate	+	+	+	+
Methanol, ethanol, <i>n</i> -propanol	+	+	+	+
α-Ketoglutarate	+	–	+	+
L(+)-Ascorbate	+	–	+	nd
Methylamines	+	+	+	nd
Carbonyl sulfide, carbon disulfide, dimethylsulfide, methanesulfide, (under anoxic conditions producing H ₂ S)	nd	nd	nd	+
Thiophene-2 carboxylate				
<i>N</i> -Caproate	+	d	–	nd
Citrate	+	–	D	+
Lactate	+	–	+	nd
Malonate	d	–	+	nd
Lactose, sorbose, raffinose, rhamnose	–	–	–	–
Fructose	+	–	d	+
Glucose (including weak or after adaptation)	+	–	d	+
Mannose	d	–	d	+
Sucrose	D	–	–	+
Alanine	–	+	+	+
Arginine, L-aspartate, L-phenylalanine, isoleucine, threonine, histidine	–	–	–	–
D-Glutamine	+	–	–	nd

(continued)

TABLE BXII.α.191. (cont.)

Characteristic	<i>X. autotrophicus</i>	<i>X. agilis</i>	<i>X. flavus</i>	<i>X. tagetidis</i>
<i>Resistant to:</i>				
Ampicillin, chloramphenicol	—	D	—	+
Penicillin, tetracycline	—	D	—	—
Erythromycin	—	+	—	nd
Nitrate reduced to nitrite	+	—	+	Weak + (— on API 20)
Tetrathionate to thiosulfate	+	+	+	+
Tetrazolium salts reduced	+	nd	—	nd
Lecithinase, deoxyribonuclease activities	—	nd	—	nd
Catalase, phosphate oxidase	+	+	+	nd
Urease activity	d	nd	—	nd
Tyrosinase activity	+	nd	—	nd
Indole production	—	nd	—	—
Litmus milk (alkaline reaction only change)	+	nd	+	nd
Voges-Proskauer test	—	—	—	nd
H ₂ S production	—	—	—	— (+ thiosulfate thiophene)
Gelatin liquefaction	—	nd	—	nd
Hydrolysis of Tween, starch, casein, or cellulose	—	—	—	—
Main component (60–90%) of fatty acid is 11-octadecenoic acid (cis)	+	+	+	nd
Salicin, β-hydroxybutyrate tartrate	—	nd	—	nd
Mol% G + C of DNA	69–70 (<i>T_m</i>); (65–68) ^f	66 ± 1 (Bd)	69 (<i>T_m</i>); (68) ^f	67 ± 1 (<i>Sp</i>); 71 ± 3 (<i>T_m</i>)

^aSymbols: see standard definitions; nd, not determined.

^bOccasionally as long as 8.0 μm.

^cOccasionally as long as 5.0 μm.

^dSome white/whitish strains produce only minor amount.

^eAmounts range from copious to small; slime contains acid sugars.

^fValues in parenthesis are from De Smedt et al. (1980); *T_m*, obtained by buoyant density method; Bd, value obtained by thermal denaturation; *Sp*, value obtained by spectrometric assay.

Other Organisms

1. *Xanthobacter methylooxidans*^a

The species name “*X. methylooxidans*” has been proposed for the facultatively methylotrophic bacterial strain 32P (Doronina et al., 1996c). It is characterized by a high content of *cis*-vaccenic (55–75%) and lactobacillic (13–17%) acids. It does not require vitamins, forms indole, ammonia, and hydrogen sulfide, liquefies gelatin, and utilizes formate, methylamine, dimethylformamide, dimethylacetamide, and dimethyl sulfoxide as carbon and energy sources. The oxidation of methanol proceeds with the participation of the corresponding dehydrogenases via formaldehyde and formate to CO₂, which is assimilated via the ribulose biphosphate pathway. Strain 32P oxidizes H₂ and fix N₂. The DNA–DNA hybridization values of strain 32P with type strains belonging to the genus *Xanthobacter* (*X. agilis* DSM 3770, *X. flavus* DSM 338, and *X. autotrophicus* DSM 432) did not exceed 25%; this, together with its phenotypic peculiarities, allowed strain 32P to be classified as a “new species”, however, without being validly described and named).

The mol% G + C of the DNA is: 69.5 (*T_m*).

Deposited strain: 32P.

2. Several isolates have been described either as strains without attributing them to a genus, but that resemble *Xanthobacter* strongly in their properties, or were just described as strains of *Xanthobacter* but without assignment to a species. Many autotrophically isolated strains of *X. autotrophicus* from various habitats can be obtained from J. Wiegell. The others

strains below should be available from the corresponding authors; unfortunately, the strains have not been deposited in public culture collections. Some of the early isolated and interesting strains are:

- Sixteen strains isolated on alkenes (De Bont and Leijten, 1976)
- Mycobacterium butanitricans* (Coty, 1967)
- Group IV, 10 strains (Kouno and Ozaki, 1975)
- Strains N61 and N63 (Jensen and Holm, 1975)
- Strains PY 2 (Ensign, personal communication: *X. autotrophicus*; one of the best studied strains in respect to carbon metabolism; containing coenzyme M) PY10, By2 (van Ginkel and DeBont, 1986; van Ginkel et al., 1986) utilizing alkanes under epoxide formation
- Strain 124X (ATCC 49450), degrading 4-hydroxyphenylacetate (Van den Tweel et al., 1986) and styrene (Hartmans et al., 1989)
- Strain MAB2 (ATCC 48876) (Villarreal et al., 1991) and patent strain NW11 (ATCC 53272), (Robinson and Stipanovic, 1989) polysaccharide producing strains for use in oil recovery
- Strain 25a (Meijer et al., 1990)
- Strain Cimw 99 (Smith et al., 1991) degrading 3-chloro-2-methylpropionic acid
- Strain CP (Ditzelmüller et al., 1989) degrading chlorophenols and 2,4-dichlorophenylacetic acid

Family IX. *Methylobacteriaceae* fam. nov.

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Meth.yl.o.bac.te.ri.a' ce. ae. M.L. neut. n. *Methylobacterium* type genus of the family; *-aceae* ending to denote family; M.L. fem. pl. n. *Methylobacteriaceae* the *Methylobacterium* family.

The family *Methylobacteriaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains the genera *Methylobacterium* (type genus) and *Microvirga*. *Microvirga* was proposed after the cut-off date for inclusion in this volume (June 30, 2001) and is not described here (see Kanso and Patel (2003)).

Chemoorganotrophic; *Methylobacterium* is facultatively methylotrophic. Form pink colonies.

Type genus: **Methylobacterium** Patt, Cole and Hanson 1976, 228 emend. Green and Bousfield 1983, 876.

Genus I. *Methylobacterium* Patt, Cole and Hanson 1976, 228^{AL} emend. Green and Bousfield 1983, 876

PETER N. GREEN

Meth.yl.o.bac.te'ri.um. M.L. n. *methyl* the methyl radical; Gr. n. *bacterion* a small rod; M.L. neut. n. *Methylobacterium* methyl bacterium.

Rods 0.8–1.2 × 1.0–8.0 µm that occasionally branch and/or exhibit polar growth. **Form** pink to orange-red colonies on methanol salts agar. **Stain** Gram negative to Gram variable; Gram-negative cell wall type. **Motile** by means of a single polar, subpolar, or lateral flagellum. **Aerobic**, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. **Mesophilic**. **Chemoorganotrophic and facultatively methylotrophic**, able to grow with formaldehyde (often at micromolar concentrations), formate, and methanol; some strains grow on methylated amines. Widely distributed in nature.

The mol% G + C of the DNA is: 68.0–72.4.

Type species: **Methylobacterium organophilum** Patt, Cole and Hanson 1976, 228.

FURTHER DESCRIPTIVE INFORMATION

Morphology and cell structure All *Methylobacterium* strains are rods (0.8–1.2 × 1.0–8.0 µm) that occur singly or occasionally in rosettes (Heumann, 1962; Patt et al., 1974). They are often branched or pleomorphic, especially in older stationary-phase cultures (Fig. BXII.α.245). There is some evidence to suggest that they exhibit polar growth or a budding morphology (L.B. Perry, unpublished observations). All strains are motile by a single polar, subpolar or lateral flagellum, although some strains are not vigorously motile. Cells often contain large sudanophilic inclusions (poly-β-hydroxybutyrate) and sometimes volutin granules. They are Gram negative, although many strains stain as Gram variable. Representative strains have a multilayered cell wall structure and the type of citrate synthase (Green and Bousfield, 1982) characteristic of Gram-negative bacteria.

Cultural features Most strains grow slowly but a few grow poorly or not at all on nutrient agar. After 7 d incubation at 30°C, colonies on GP agar are 1–3 mm in diameter and pale pink to bright orange-red, whereas colonies on MMS agar are a more uniform pale pink. The pigment is nondiffusible, nonfluorescent, and is a carotenoid (Ito and Iizuka, 1971; Downs and Harrison, 1974; Urakami et al., 1993). In static liquid media, most strains form a pink surface ring and/or pellicle.

The optimal growth temperature for all *Methylobacterium* strains is in the range 25–30°C. Some strains will grow at 15°C or less, and some will grow at or above 37°C. Although growth is optimal around neutrality, some strains can grow at pH 4 and some at pH 10.

Pigments All strains contain carotenoid pigments that have absorption maxima at 465, 495, and 525 nm (Urakami et al., 1993). Sato (1978), Sato and Shimizu (1979), and Nishimura et al. (1981) have shown that strains of *Methylobacterium* can form bacteriochlorophyll *a* under specific cultural conditions, suggesting a common link with the phototrophs in their ancestry.

Metabolism and methylotrophy All strains are strict aerobes and are catalase and oxidase (often weakly) positive. They are chemoorganotrophs and facultative methylotrophs, capable of growth on a variety of C₁ compounds. All grow on formaldehyde (often at micromolar concentrations), formate, and methanol; some grow on methylated amines. Only one species (*M. organophilum*) is reported to have utilized methane as sole carbon and energy source, but the ability has since been lost by the type (and only) strain. Methane assimilability in this organism was thought to be plasmid borne and easily lost if cultures were not maintained on an inorganic medium in a methane atmosphere (R.S. Hanson, personal communication). This led Green and Bousfield (1983) to emend the genus description of *Methylobacterium* to exclude methane assimilation as a key taxonomic criterion. Representative *Methylobacterium* strains have been reported to assimilate C₁ compounds via the icl(-) serine pathway (the type of serine C₁-assimilatory pathway used by these organisms) (Quayle, 1972; Bellion and Spain, 1976) and to have a complete tricarboxylic acid cycle when grown on complex organic substrates.

Growth factors are not required by any strain although growth of some strains is stimulated by calcium pantothenate. Most strains do not degrade or hydrolyze casein, starch, gelatin, cellulose, lecithin, or DNA. Urease is produced by all strains, and some strains have weak lipolytic activity. The enzymes β-galactosidase, L-ornithine decarboxylase, L-lysine decarboxylase, and L-arginine dihydrolase are not produced. Indole (except for *M. thiocyanatum*) and H₂S are also not produced. The methyl red and Voges–Proskauer tests are negative, although some strains reduce nitrate to nitrite.

Carbon and nitrogen sources The following compounds were used as carbon sources by most (≥95%) strains of *Methylobacterium*: glycerol, malonate, succinate, fumarate, α-ketoglutarate, DL-lactate, DL-malate, acetate, pyruvate, propylene glycol, ethanol, methanol, and formate. Some strains (see Green and

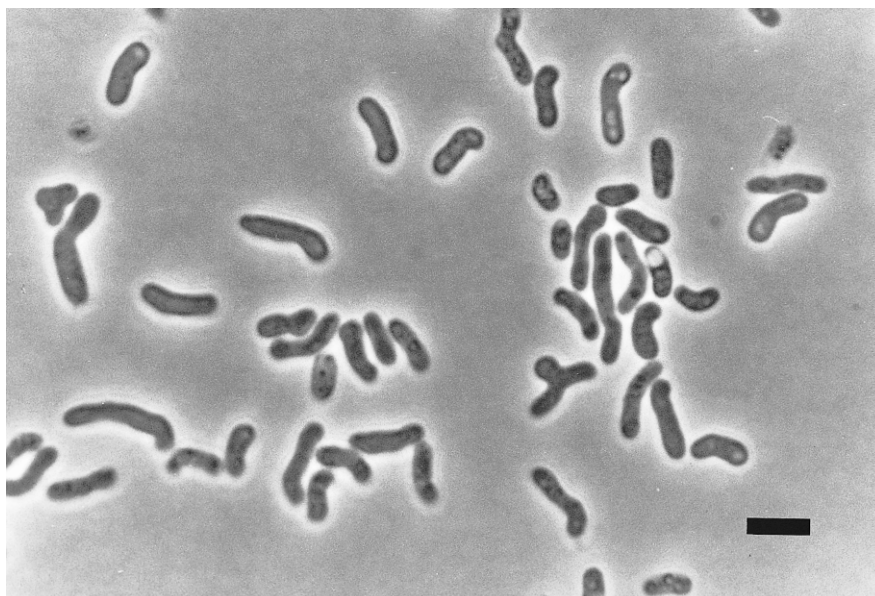


FIGURE BXII.α.245. Phase-contrast micrograph of *Methylobacterium* spp. Bar = 2 μm.

Bousfield, 1982; Urakami et al., 1993, and Table BXII.α.192) can also utilize L-arabinose, D-xylose, D-fucose, D-glucose, D-galactose, D-fructose, L-aspartate, L-glutamate, adipate, pimelate, sebacate, azelate, suberate, D-tartrate, citrate, citraconate, saccharate, monomethylamine, dimethylamine, trimethylamine, trimethylamine-*N*-oxide, ethanolamine, butylamine, formamide, *N*-methylformamide, dimethylglycine, betaine, tetramethylammonium hydroxide (TMAH), *N,N*-dimethylformamide (DMF), chloromethane, and dichloromethane. None of the strains appear to use any of the disaccharides or sugar alcohols examined (except for glycerol) (Green and Bousfield, 1982) or any of the following as sole carbon and energy source: propionate, DL-arginine, L-valine, glycine, geraniol, tryptamine, histamine, putrescine, *m*-hydroxybenzoate, testosterone, sarcosine, phenol, thiourea, tetramethyl urea, hexane, or benzene. Oxidative production of acid from sugars was variable among strains Urakami et al. (1993).

Ammonia, nitrate, and urea can serve as nitrogen sources (Urakami and Komagata, 1984). *M. thiocyanatum* can utilize thiocyanate or cyanate as sole source of nitrogen for growth (Wood et al., 1998).

Fatty acid composition The fatty acid composition of *Methylobacterium* strains is comprised largely (around 70–90%) of C_{18:1} acid with small amounts of C_{16:1}, C_{19:0 cyclo}, and C_{14:0 3OH} hydroxy acid. Some strains contain hopanoids and small amounts of squalene. Phospholipids of all strains included large amounts of cardiolipin (diphosphatidylglycerol), phosphatidylethanolamine, phosphatidylcholine, and a small amount of phosphatidylglycerol (Urakami et al., 1993). The major isoprenoid quinone components are ubiquinones with 10 isoprene units (Urakami and Komagata, 1979). DNA base composition is 68.0–72.4 mol% G + C (Hood et al., 1987).

Antibiotic sensitivity Most strains are sensitive to the chemotherapeutic agents kanamycin, gentamicin, albamycin T, streptomycin, framycetin, and especially the tetracyclines, whereas most are resistant to cephalothin, nalidixic acid, penicillin, bacitracin, carbenicillin, colistin sulfate, polymyxin B, and nitrofurantoin.

Habitats and sources Being ubiquitous in nature and a common airborne organism, strains of *Methylobacterium* spp. are found in a wide variety of environmental, industrial, and even occasionally clinical environments, mainly as part of a transient flora or as chance contaminants. Their ability to scavenge trace amounts of nitrogen and to resist a certain degree of desiccation contributes to their survival in hostile environments. Some strains have been shown to exhibit resistance to gamma irradiation 10–40 times higher than that exhibited by many other Gram-negative bacteria (Ito and Iizuka, 1971). *M. thiocyanatum* (Wood et al., 1998) has been shown to tolerate high (≥50 mM) thiocyanate or cyanate. Other *Methylobacterium* strains have not been tested for this feature.

ENRICHMENT AND ISOLATION PROCEDURES

Because of the ability of *Methylobacterium* spp. to grow on methanol as sole carbon and energy source, and because of their characteristic pigmentation, these organisms are relatively easy to isolate. Methanol mineral salts (MMS)¹ medium is a suitable selective medium for *Methylobacterium*.

Although many *Methylobacterium* strains can grow between 5°C and 37°C, all grow well at 30°C, and thus 25–30°C can be used for all isolation and subsequent growth experiments. These organisms are fairly slow growers, often taking 2–3 d at 30°C to produce clearly visible colonies or confluent growth, and often taking more than 7 d for colonies to reach their maximum size of 1–3 mm in diameter. Growth is sometimes more luxuriant, with

1. Methanol mineral salts medium consists of (per liter water): K₂HPO₄, 1.20 g; KH₂PO₄, 0.62 g; CaCl₂·6H₂O, 0.05 g; MgSO₄·7H₂O, 0.20 g; NaCl, 0.10 g; FeCl₃·6H₂O, 1.0 mg; (NH₄)₂SO₄, 0.5 mg; CuSO₄·5H₂O, 5.0 mg; MnSO₄·5H₂O, 10.0 mg; Na₂MoO₄·2H₂O, 10.0 mg; H₃BO₃, 10.0 mg; ZnSO₄·7H₂O, 70.0 mg; CoCl₂·6H₂O, 5.0 mg. The medium is sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. A filter-sterilized vitamin solution (Colby and Zatman, 1973) is added if required, along with 0.1–0.2% v/v sterile methanol. The pH of the medium is adjusted to pH 7.0. A solidified medium (MMS agar) is prepared by the addition of 1.5–2% Oxoid purified agar before autoclaving. No *Methylobacterium* strain isolated to date has been shown to have an obligate requirement for vitamins or other added growth factors.

TABLE BXII.α.192. Substrates utilized as sole carbon source to differentiate strains of *Methylobacterium*^{a,b}

Characteristic	<i>M. organophilum</i>	<i>M. aminovorans</i>	<i>M. chloromethanicum</i> ^c	<i>M. dichloromethanicum</i> ^d	<i>M. extorquens</i>	<i>M. fujisawaense</i>	<i>M. mesophilicum</i>	<i>M. radiotolerans</i>	<i>M. rhodesianum</i>	<i>M. rhodinum</i>	<i>M. thiocyanatum</i> ^e	<i>M. zatmanii</i>
D-Glucose	+	—	—	—	—	V	V	+	—	V	+	—
D-Fucose	—	—	nd	—	—	+	+	+	—	—	nd	—
D-Xylose	—	—	—	—	—	+	+	+	—	—	nd	—
L-Arabinose	—	—	—	—	—	+	+	+	—	—	V	—
Fructose	+	+	—	+	—	V	—	—	+	+	+	+
L-Aspartate/L-Glutamate	—	+	—	+	V	+	+	+	V	+	+	—
Citrate	—	—	—	—	—	+	+	+	—	+	+	—
Sebacate ^g	—	—	nd	—	—	+	V	+	—	—	nd	—
Acetate	+	+	nd	+	+	V	—	+	+	+	+	+
Betaine	—	+	—	+	+	—	—	+	+	+	nd	—
Methylamine	+	+	+	+	+	—	—	—	+	+	+	+
Trimethylamine	+	+	—	—	—	—	—	—	—	—	—	V
Methane	V	—	—	—	—	—	—	—	—	—	nd	—
Tetramethylammonium hydroxide	—	+	nd	nd	—	—	—	—	—	—	nd	—
N,N-dimethylformamide	—	+	—	—	—	—	—	—	—	—	nd	—
Chloromethane	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Dichloromethane	nd	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd
Growth on peptone-rich nutrient agar ^h	+	+	—	+	V	+	—	+	+	+	+	+

^aSymbols: +, utilized as substrate; —, not utilized; V, variable result; nd, no data.^bOwing to the slow growth of some strains on certain substrates, carbon utilization tests were read after 14 days incubation.^cData from McDonald et al. (2001).^dData from Doronina et al. (2000b).^eData from Wood et al. (1998).^fTested for glutamate only.^gMost strains which utilize sebacate can also utilize pimelate, suberate, azelate, and adipate.^hNutrient agar, e.g., Oxoid cm55.

a deeper pigmentation, on Glycerol–Peptone (GP) agar². Although this medium is useful for subculturing stocks of pure *Methylobacterium* spp., it is less suitable for enrichment than the MMS medium, as other rapidly growing heterotrophs present in the sample can overgrow the pink-pigmented facultative methylotrophic (PPFM) bacteria. The use of certain antibiotics in selective media (see Further Descriptive Information, this chapter) can also be considered, as can the use of individual carbon sources, for the isolation of specific groups or species of *Methylobacterium*.

If MMS agar is used as a selective medium, the vast majority of the pink colonies which reach diameters of more than 1 mm will be strains of PPFM organisms. Pink methylotrophic yeasts are not uncommon, but bacterial pink methylotrophs other than *Methylobacterium* species are rare. Growth of PPFM organisms in liquid media is almost always characterized by a surface ring and/or thin pellicle, indicative of their aerobic nature.

When attempting to isolate PPFM strains from leaf surfaces, a leaf impression technique, using one of the above media, is recommended. Homogenization of whole leaves or embedding of leaves in molten agar are alternatives, although they are not as successful as the impression technique.

If fungal contamination is a problem with samples from par-

ticular habitats when attempting to isolate strains of PPFM, 20 µg/ml of cycloheximide can be added to the medium.

MAINTENANCE PROCEDURES

Strains of *Methylobacterium* can be maintained short term (2–4 weeks) on GP or methanol salts agar. For long-term preservation organisms can be stored at –70°C or in/over liquid nitrogen, using either of the above liquid growth media supplemented with 10% v/v glycerol; alternatively, the organisms can easily be lyophilized. Strains isolated on methane and dichloromethane must be maintained on a salts medium containing these compounds as sole carbon source or their ability to utilize these substrates may be lost.

PROCEDURES FOR TESTING SPECIAL CHARACTERS

Because the nine species of *Methylobacterium* are differentiated mainly by the pattern of compounds they utilize as sole carbon and energy source, care should be taken to standardize such tests since they are notoriously difficult to duplicate between laboratories. All carbon utilization tests shown in Table BXII.α.192 were carried out as described by Green and Bousfield (1982), who used faintly turbid suspensions of cells that had been thrice washed in sterile saline, to inoculate media. All tests were read only after 14 days incubation at 30°C, and growth was compared to a negative control with no added carbon source. This long incubation time was necessary to allow for slow growth on certain compounds. Tests for biochemical, physiological, and morpho-

2. Glycerol–Peptone Agar consists of (g/l): agar, 15.0; glycerol, 10.0; peptone (Difco), 10.0. The pH is adjusted to pH 7.0.

logical features as well as calculations of DNA base ratios are described by Green and Bousfield (1982) and Green (1992).

TAXONOMIC COMMENTS

Many of the strains which comprise the genus *Methylobacterium* have a checkered taxonomic history (Green and Bousfield, 1981) and based on their Gram variability, occasional cellular pleomorphism, and flagellar arrangements were previously included in the following genera: *Bacillus*, *Vibrio*, *Pseudomonas*, *Flavobacterium*, "*Protaminobacter*", *Mycoplana*, *Protomonas*, and *Methylobacter-*

ium. Taxonomic studies (Wolfrum et al., 1986; Hood et al., 1987, 1988; Urakami et al., 1993) have shown the genus to be heterogeneous, with 12 validly published species to date (Green, 1992; Urakami et al., 1993; Wood et al., 1998; Doronina et al., 2000b; McDonald et al., 2001), as well as several unassigned strains representing centers of genetic variation which may equate to new taxa. Recent DNA-rRNA homology studies by Dreyfus et al. (1988) have placed *Methylobacterium* in the rRNA superfamily IV of De Ley (1978), along with other members of the *Agrobacterium-Rhizobium* complex.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *METHYLOBACTERIUM*

Species within the genus *Methylobacterium* are differentiated mainly by the pattern of compounds they utilize as sole carbon and energy source (see Table BXII.α.192).

List of species of the genus *Methylobacterium*

1. ***Methylobacterium organophilum*** Patt, Cole and Hanson 1976, 228^{AL}

orga.no'phi.lum. Gr. n. *organo* organ, living bodies; Gr. adj. *philos* loving; Gr. adj. *organophilus* intended to mean preferring complex carbon sources.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. Originally reported as being able to utilize methane as sole source of carbon and energy (Patt et al., 1974), but this feature has subsequently been lost (Green and Bousfield, 1982). The type strain was isolated from lake sediment.

The mol% G + C of the DNA is: 70.5 (T_m); 71.1 (Bd).

Type strain: XX, ATCC 27886, DSM 760, NCIMB 11278.

GenBank accession number (16S rRNA): D32226.

2. ***Methylobacterium aminovorans*** Urakami, Araki, Suzuki and Komagata 1993, 510^{VP}

a.mi.no'vo.rans. N.L. n. *aminus* amine; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *aminovorans* amine digesting.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from soil.

The mol% G + C of the DNA is: 68.0 (HPLC).

Type strain: TH15, JCM 8240, NCIMB 13343.

3. ***Methylobacterium chloromethanicum*** McDonald, Doronina, Trotsenko, McAnulla and Marrell 2001, 121^{VP}

chlo.ro.me.tha'ni.cum. N.L. n. *chloromethanicum* chloromethane utilizing.

Characteristics are those given for the genus and/or the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from soil at the Nizheksk petrochemical factory, Tatarstan, Russia.

The mol% G + C of the DNA is: 64.4 (T_m).

Type strain: CM4, NCIMB 13688.

GenBank accession number (16S rRNA): AF198624.

4. ***Methylobacterium dichloromethanicum*** Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2000c, 1953^{VP} (Effective publication: Doronina, Trotsenko, Tourova, Kuznetsov and Leisinger 2000b, 216.)

di.chlo.ro.meth.an'ic.um. M.L. n. *methanum* methane; L. pref. *di* two; M.L. adj. *dichloromethanicum* dichloromethane utilizing.

Characteristics are those given for the genus and/or the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from activated sludge.

The mol% G + C of the DNA is: 67.1 (T_m).

Type strain: DM4, DSM 6343.

GenBank accession number (16S rRNA): AF227128.

5. ***Methylobacterium extorquens*** (Bassalik 1913) Bousfield and Green 1985, 209^{VP} (*Protomonas extorquens* (Bassalik 1913) Urakami and Komagata 1984, 198; *Bacillus extorquens* Bassalik 1913, 258.)

ex.tor'quens. L. part. adj. *extorquens* twisting out.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from earthworm excreta in 1949 by L. Janota-Bassalik. Bassalik's original strain is no longer extant.

The mol% G + C of the DNA is: 69.4 (Bd).

Type strain: ATCC 43645, DSM 1337, NCIMB 9399.

GenBank accession number (16S rRNA): L20847, D32224.

6. ***Methylobacterium fujisawaense*** Green, Bousfield and Hood 1988, 124^{VP}

fu.ji.sa'wa.en.se. N.L. neut. adj. *fujisawaense* coming from the Fujisawa region of Japan.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192.

The mol% G + C of the DNA is: 70.8–71.8 (Bd).

Type strain: 0-31 (Kouno and Ozaki 1975), ATCC 43884, DSM 5686, NCIMB 12417.

7. ***Methylobacterium mesophilicum*** (Austin and Goodfellow 1979) Green and Bousfield 1983, 876^{VP} (*Pseudomonas mesophilica* Austin and Goodfellow 1979, 377.)

me.so.phi'li.cum. Gr. n. *meson* the middle; Gr. adj. *philikos* friendly; N.L. neut. adj. *mesophilicum* friendly to the middle, because of its preference for moderate temperatures.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from *Lolium perenne* leaves.

The mol% G + C of the DNA is: 69.9 (Bd).

Type strain: A47, ATCC 29983, DSM 1708, NCIMB 11561.

GenBank accession number (16S rRNA): D32225.

8. ***Methylobacterium radiotolerans*** (Ito and Iizuka 1971) Green and Bousfield 1983, 876^{VP} (*Pseudomonas radiora* Ito and Iizuka 1971, 1568.)

ra.di.o.to'le.rans. Eng. pref. *radio* pertaining to radiation; L. part. adj. *tolerans* tolerating; N.L. part. adj. *radiotolerans* tolerating radiation.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from rice grains.

The mol% G + C of the DNA is: 72.3 (Bd).

Type strain: 0-1, ATCC 27329, DSM 1819, NCIMB 10815.
GenBank accession number (16S rRNA): D32227.

9. **Methylobacterium rhodesianum** Green, Bousfield and Hood 1988, 124^{VP}
rho' de.si.an.um. M.L. neut. adj. *rhodesianum* named after the British taxonomist Muriel Rhodes-Roberts.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from a fermentor operating with formaldehyde as sole carbon source.

The mol% G + C of the DNA is: 69.8–71.2 (Bd).

Type strain: *Pseudomonas* strain 1, ATCC 43882, DSM 5687, NCIMB 12249.

GenBank accession number (16S rRNA): L20850.

10. **Methylobacterium rhodinum** (Heumann 1962) Green and Bousfield 1983, 876^{VP} (*Pseudomonas rhodos* Heumann 1962, 342.)
ro.di' num. N.L. neut. adj. *rhodinum* rosy, because of its pink color.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated by Heumann (1962) from *Alnus* rhizosphere.

The mol% G + C of the DNA is: 71.8 (Bd).

Type strain: ATCC 14821, DSM 2163, NCIMB 1942.

GenBank accession number (16S rRNA): L20849, D32229.

11. **Methylobacterium thiocyanatum** Wood, Kelly, McDonald, Jordan, Morgan, Khan, Murrell and Borodina 1999, 341^{VP} (Effective publication: Wood, Kelly, McDonald, Jordan, Morgan, Khan, Murrell and Borodina 1998, 157.)
thio.cy.an.a'tum. M.L. n. *thiocyanatum* using thiocyanate.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. Able to tolerate high (≥ 50 mM) thiocyanate and/or cyanate and utilize these compounds as sole nitrogen source for growth. The type strain was isolated from soil in the rhizosphere of *Allium aflatumense*.

The mol% G + C of the DNA is: 69.8–71.0 (T_m).

Type strain: ALL/SCN-P, ATCC 700647, DSM 11490.

GenBank accession number (16S rRNA): U58018.

12. **Methylobacterium zatmanii** Green, Bousfield and Hood 1988, 124^{VP}
zat.ma'ni.i. M.L. gen. n. *zatmanii* named after the British biochemist L.J. Zatman.

Characteristics are those given for the genus and for the carbon utilization spectra shown in Table BXII.α.192. The type strain was isolated from a fermentor operating with formaldehyde as sole carbon source.

The mol% G + C of the DNA is: 69.4–70.3 (Bd).

Type strain: 125, ATCC 43883, DSM 5688, NCIMB 12243.

GenBank accession number (16S rRNA): L20804.

Family X. Rhodobiaceae fam. nov. *

GEORGE M. GARRITY, JULIA A. BELL AND TIMOTHY LILBURN

Rho.do.bi.a'ce.ae. M.L. n. *Rhodobium* type genus of the family; -aceae ending to denote family;
M.L. fem. pl. n. *Rhodobiaceae* the *Rhodobium* family.

The family *Rhodobiaceae* was circumscribed for this volume on the basis of phylogenetic analysis of 16S rRNA sequences; the family contains *Rhodobium* (type genus).

Grow phototrophically in the light and chemotrophically in

the dark. Photosynthetic membranes present as lamellar stacks. Require NaCl for growth.

Type genus: **Rhodobium** Hiraishi, Urata and Satoh 1995d, 230.

Genus I. Rhodobium Hiraishi, Urata and Satoh 1995d, 230^{VP}

JOHANNES F. IMHOFF AND AKIRA HIRAISHI

Rho.do'bi.um. Gr. n. *rhodon* the rose; Gr. n. *bios* life; M.L. n. *Rhodobium* red life.

Cells are ovoid to rod shaped, motile by means of polar, subpolar or randomly distributed flagella; and cells multiply by budding and asymmetric cell division. Rosette formation is rare. **Gram negative and belong to the Alphaproteobacteria.** Phototrophically grown cells contain **internal photosynthetic membranes as lamellar stacks parallel to the cytoplasmic membrane.** Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. **Straight-chain saturated and monounsaturated C_{18:1} and C_{18:0} are the major components of cellular fatty acids, with the former being predominant.** Ubiquinones and menaquinones with 10 isoprene units (Q-10 and MK-10) are present as major quinones.

Preferred growth is photoheterotrophically under anoxic conditions in the light with simple organic substrates as carbon and electron sources. **Chemotrophic growth is possible under oxic conditions in the dark.** Dark fermentative growth may also be possible. Growth factors are required. **Mesophilic, marine bacteria that require NaCl for growth** and grow at seawater salinity and also at slightly higher salt concentrations.

The mol% G + C of the DNA is: 61.5–65.7.

Type species: **Rhodobium orientis** Hiraishi, Urata and Satoh 1995d, 230.

FURTHER DESCRIPTIVE INFORMATION

Cells of *Rhodobium* multiply by budding or asymmetric cell division, and show polar growth (Fig. BXII.α.246), as do other species of phototrophic bacteria in the alpha-2 *Proteobacteria*. They

*Editorial Note: As this subvolume went to press, *Roseospirillum* was moved from the Family *Rhodobiaceae* to the Family *Rhodospirillaceae*. Placement in either family is tentative.

form sessile buds, but prosthecae between mother and daughter cells, which are characteristic for other phototrophic budding bacteria, are only found in *R. orientis*. Rosette-like cell aggregates are occasionally found in *R. orientis* (Fig. BXII.α.246) but not in *R. marinum*. In particular, *R. marinum* contains very active and agile motile cells.

A quantitative analysis of the carotenoids of *R. marinum* revealed that spirilloxanthin and rhodovibrin constituted 65% and 24%, respectively, of the total content, whereas anhydrorhodovibrin, monodemethylated spirilloxanthin, and lycopene were minor components (Imhoff, 1983). Absorption spectra of living cells or membrane preparations of *R. orientis* show major absorption maxima at around 800 and 870 nm, suggesting that the core light-harvesting complex (LH I), together with the photosynthetic reaction center and a peripheral light-harvesting complex (LH II), are present. Cells and membrane preparations of *R. marinum*, however, exhibit an absorption maximum at 883 nm and have very low absorption at 800 nm, similar to *Rhodospirillum rubrum*. This is due to the presence of LH I as the only antenna complex. The core antenna complex of *R. marinum* consists of 24 polypeptides with 24 bacteriochlorophyll *a* molecules, and the B880 complex exhibits a six-fold symmetry of the ring-like structure (Meckenstock et al., 1994).

Major polar lipid components of *R. marinum* are phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, cardiolipin, and an ornithine lipid. Also unusually high proportions of a sulfolipid, most probably sulfoquinovosyl diglyceride, and an unidentified aminolipid are produced (Imhoff, 1983).

Based on 16S rDNA sequence information, *Rhodobium* species represent a deeply branching line of descent in the group of bacteria known as the alpha-2 *Proteobacteria*, where they are most closely related to representatives of *Rhizobium* and *Sinorhizobium*. 16S rDNA sequence analysis indicates that *R. orientis* and *R. marinum* share 93% similarity, a relatively low value for two species of the same genus. In addition, *R. marinum* has a shorter length

of 16S rDNA than *R. orientis*. This is due to a 21-base deletion in the conserved region of the 16S rRNA between positions 1256 and 1281 (according to the *Escherichia coli* numbering system), like the deletion found in the bacteria known as the alpha-2 group of the *Proteobacteria*. This sequence deletion is a good marker for differentiation of *R. marinum* from *R. orientis* and other species of the group of bacteria known as the alpha-2 *Proteobacteria*.

Rhodobium species occur in marine and hypersaline environments as their natural habitats. Strains of *R. orientis* have been isolated from tidal pools and coastal seashore of Japan. *R. marinum* appears to be one of the most common species of phototrophic purple bacteria in marine environments. Strains of this species have been isolated from diverse sites in the world, including a splash water pond at the Adriatic shore near Split (Yugoslavia), Solar Lake (Sinai), an intertidal flat near Inverary in Scotland (Imhoff, 1983), a microbial mat at Laguna Guerrero Negro in Mexico (Mangels et al., 1986), and surface seawater at the coast of Japan (A. Hiraishi, unpublished).

As with other members of the purple nonsulfur bacteria (PNSB), growth with limited concentrations of ammonium salts or amino acids as nitrogen sources leads to extensive H₂ photoevolution due to nitrogenase activity.

ENRICHMENT AND ISOLATION PROCEDURES

The methods and media used routinely for the cultivation of phototrophic PNSB (Imhoff, 1988; Imhoff and Trüper, 1992) can be employed for isolation and enrichment of *Rhodobium* species, if NaCl is added at appropriate concentrations. Media with 3–5% NaCl and pH 6.8–7.0 are suitable for *Rhodobium* species (Imhoff and Trüper, 1976; Imhoff, 1983; Hiraishi et al., 1995d).

MAINTENANCE PROCEDURES

Cultures are well preserved in liquid nitrogen, by lyophilization, or at –80°C in a mechanical freezer.

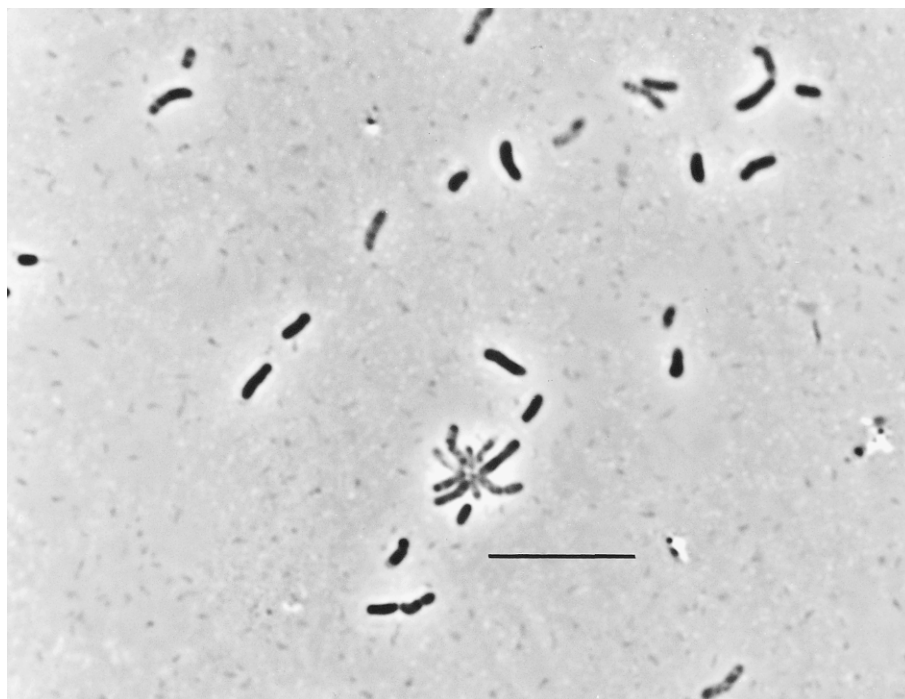


FIGURE BXII.α.246. Phase-contrast photomicrograph showing general cell morphology of *Rhodobium orientis* (strain MB 312). Bar = 10 μm.

DIFFERENTIATION OF THE GENUS *RHODOBIUM* FROM OTHER GENERA

The genus *Rhodobium* consists of species of the PNSB that are truly marine or slightly halophilic bacteria, multiply by budding or asymmetric cell division, and contain internal photosynthetic membrane systems of the lamellar type. In these respects, *Rhodobium* species are similar to other bacteria in the group of phototrophic bacteria belonging to the alpha-2 *Proteobacteria*, such as *Rhodoplanes*, *Blastochloris*, and *Rhodopseudomonas*. The genus *Rhodobium* is differentiated from the latter genera by its phylogenetic position, natural habitats, and chemotaxonomic characteristics. Major differentiating properties between *Rhodobium* and other phototrophic alpha-2 *Proteobacteria* are shown in Tables 3 (pp. 125–126) and 4 (p. 127) of the introductory chapter “Anoxygenic Phototrophic Purple Bacteria”, Volume 2, Part A. The phylogenetic relationships of these bacteria based on 16S rDNA sequences are shown in Fig. 2 (p. 128) of that same chapter.

TAXONOMIC COMMENTS

At present, the genus *Rhodobium* consists of two species, *R. orientis* and *R. marinum*. After tentative assignment to *Rhodopseudomonas*

palustris (Imhoff and Trüper, 1976), primarily based on morphological and physiological characteristics, the latter species was described as *Rhodopseudomonas marina* (Imhoff, 1983). Later, phylogenetic analysis based on 16S rDNA sequences revealed that this species was far distant from *Rhodopseudomonas palustris*, the type species of the genus *Rhodopseudomonas*, but closely related to new isolates from marine environments (Hiraishi et al., 1995d). These new isolates were assigned to the new genus *Rhodobium* and described as the type species of this genus, *Rhodobium orientis*, and *Rhodopseudomonas marina* was transferred to this genus as *Rhodobium marinum* (Hiraishi et al., 1995d).

Mangels et al. (1986) isolated a N₂-fixing marine strain of PNSB and designated it “*Rhodopseudomonas marina* var. *agilis*”. Upon reinvestigation, no differences in physiological properties and 16S rDNA sequence between the type strain of *Rhodobium marinum* and “*Rhodopseudomonas marina* var. *agilis*” were found (Hiraishi et al., 1995d), confirming the phenotypic and genotypic coherency of the two organisms at the species level. Therefore, “var. *agilis*” isolates should be considered as strains of *Rhodobium marinum*.

DIFFERENTIATION OF THE SPECIES OF THE GENUS *RHODOBIUM*

Major differentiating properties between *Rhodobium* species are shown in Tables BXII.α.193 and BXII.α.194.

List of species of the genus *Rhodobium*1. *Rhodobium orientis* Hiraishi, Urata and Satoh 1995d, 230^{VP} *o.ri.en'tis*. L. part. adj. *orientis* of the orient.

Cells are ovoid to rod-shaped, 0.7–0.9 × 1.5–3.2 μm, motile by means of polar, subpolar, and randomly distributed flagella; cells multiply by budding and asymmetric cell division (Fig. BXII.α.246). Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane (Fig. BXII.α.247). Phototrophically grown liquid cultures are pink-to-red, whereas aerobically grown or denitrifying cultures are faintly pink or colorless. Absorption maxima of living cells are at 377, 468, 500, 530, 591, 802, and 870 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spirilloxanthin series.

Preferred mode of growth is photoheterotrophically in the light. Photoautotrophic growth with thiosulfate, but not sulfide, as the electron donor occurs in the presence of 0.01% yeast extract. Chemotrophic growth occurs under oxic conditions in the dark at the full oxygen tension of air, and by denitrification under anoxic dark conditions with nitrate as a terminal electron acceptor. Good growth is possible with acetate, lactate, pyruvate, succinate, fumarate, malate, fructose, glucose, peptone, and yeast extract as carbon sources. Moderate growth occurs with butyrate, valerate, caproate, xylose, mannitol, and sorbitol. Formate, propionate, caprylate, citrate, tartrate, benzoate, arabinose, galactose, mannose, glycerol, methanol, ethanol, and Cas-amino acids are not utilized. Ammonium salts are used as nitrogen sources. Sulfate and thiosulfate are assimilated. Hydrolytic activities against starch, gelatin, casein, and Tween 80 are absent. Poly-β-hydroxybutyrate granules are formed as a storage material. Growth is stimulated considerably by addition of 0.05% yeast extract. Biotin and *p*-aminobenzoic acid are required as growth factors. Meso-

philic, marine bacterium with optimal growth at 30–35°C, pH 7.0–7.5 (pH range: 6.0–8.5), and in the presence of 4–5% NaCl (salinity range: 2–8% NaCl). No growth in the absence of NaCl.

Habitat: tidal seawater pools and similar marine environments.

Major quinone components are Q-10 and MK-10.

The mol% G + C of the DNA is: 65.2–65.7 (HPLC).

Type strain: MB312, ATCC 51972, DSM 11290, JCM 9337.

GenBank accession number (16S rRNA): D30792.

2. *Rhodobium marinum* (Imhoff 1984b) Hiraishi, Urata and Satoh 1995d, 320^{VP} (*Rhodopseudomonas marina* Imhoff 1984b, 270.)

ma.ri'num. L. adj. *marinum* of the sea, marine.

Cells are ovoid to rod-shaped, 0.7–0.9 × 1.0–2.5 μm, multiply by budding cell division without prostheca, and are highly motile by means of randomly distributed flagella. Rosette formation is not observed. Internal photosynthetic membranes are present as lamellae underlying and parallel to the cytoplasmic membrane. Phototrophically or fermentatively grown liquid cultures are pink-to-red, whereas aerobically grown cultures are faintly pink or colorless. Absorption maxima of living cells are at 375, 483, 516, 533, 590, 803, and 883 nm, with only a low absorption maximum around 800 nm. Photosynthetic pigments are bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spirilloxanthin series with spirilloxanthin as the dominant component.

Cells preferably grow photoheterotrophically under anoxic conditions in the light. Growth under microoxic dark conditions is also possible. Photoautotrophic growth with sulfide as a photosynthetic electron donor is poor, but the addition of 0.01% yeast extract enhances growth and sulfide tolerance. No photoautotrophic growth occurs with thio-

TABLE BXII.α.193. Differential characteristics of the species of the genus *Rhodobium*^a

Characteristic	<i>Rhodobium orientis</i>	<i>Rhodobium marinum</i>
Cell diameter (μm)	0.7–0.9	0.7–0.9
Type of budding	Sessile	Sessile
Rosette formation	– / +	–
Internal membrane system	Lamellae	Lamellae
Motility	+	+
Color of cultures	Pink to red	Pink to red
Bacteriochlorophyll	<i>a</i>	<i>a</i>
Salt requirement	4–5%	1–5%
Optimal pH	7.0–7.5	6.9–7.1
Optimal temperature	30–35	25–30
Sulfate assimilation	nd	nd
Aerobic dark growth	+	(+)
Denitrification	+	–
Fermentation of fructose	–	+
Photoautotrophic growth with	Thiosulfate	Sulfide
Growth factors	Biotin, <i>p</i> -aminobenzoic acid	nd
<i>Utilization of:</i>		
Benzoate	–	–
Citrate	–	+ / –
Formate	–	(+)
Glucose	+	+
Tartrate	–	–
Sulfide	–	(+)
Thiosulfate	+	–
Mol % G + C of the DNA	65.2–65.7 (HPLC)	62.4–64.1 (HPLC)
Cytochrome <i>c</i> ₂ size	nd	nd
Major quinones	Q-10, MK-10	Q-10, MK-10
<i>Major fatty acids</i>		
C _{14:0}		0.4
C _{16:0}		1.9
C _{16:1}		0.5
C _{18:0}		14.1
C _{18:1}		69.0

^a+, positive in most strains; –, negative in most strains; + / –, variable in different strains; nd, not determined; (+), weak growth or microaerobic growth only; (APS), via adenosine-5'-phosphosulfate; (PAPS), via 3'-phosphoadenosine-5'-phosphosulfate; (biotin) biotin is required by some strains; Q-10, ubiquinone 10; MK-10, menaquinone 10; RQ-10, rhodoquinone 10. Bd, buoyant density.

sulfate as the electron donor. Fermentative growth in the dark occurs with fructose as substrate. Denitrification is not possible. All or most strains use the following compounds as electron donors and carbon sources: acetate, propionate, butyrate, valerate, caproate, lactate, pyruvate, succinate, fumarate, malate, ascorbate, fructose, glucose, sucrose, mannitol, sorbitol, ethanol, propanol, and Casamino acids. Weak growth occurs with formate, galactose, and glycerol. Benzoate, tartrate, and methanol are not utilized. Sulfate is assimilated and, in addition, thiosulfate, tetrathionate, cysteine, methionine, glutathione, sulfite, and sulfide can serve as assimilatory sulfur sources. Ammonia, glutamate, aspartate, alanine, and urea serve as nitrogen source. Hydrolytic activities against starch, gelatin, casein, and Tween 80 are absent. Growth is stimulated considerably by adding 0.01% yeast extract.

Mesophilic marine bacterium with optimal growth at 25–30°C, pH 6.9–7.1, and 1–5% NaCl. No growth in the absence of NaCl.

Habitats: marine and moderately hypersaline waters and coastal sediments.

Major quinone components are Q-10 and MK-10.

The mol% G + C of the DNA is: 61.5–64.1 (*T_m*), 62.4–64.1 (HPLC).

Type strain: BN 126, ATCC 35675, DSM 2698.
GenBank accession number (16S rRNA): D30790.

TABLE BXII.α.194. Growth substrates of the anoxygenic phototrophic purple bacteria belonging to the genus *Rhodobium*^a

Source/donor	<i>Rhodobium orientis</i>	<i>Rhodobium marinum</i>
<i>Carbon source</i>		
Acetate	+	+
Aspartate	nd	nd
Benzoate	–	–
Butyrate	+	+
Caproate	+	+
Caprylate	–	+ / –
Citrate	–	–
Ethanol	–	+ / –
Formate	–	+
D-Fructose	+	+
Fumarate	+	+
D-Glucose	+	+
Glutamate	+ / –	nd
Glycerol	–	+ / –
Glycolate	nd	nd
Lactate	+	+ / –
Malate	+	+
Malonate	nd	nd
Mannitol	+ / –	+
Methanol	–	–
Propanol	–	+ / –
Propionate	–	+ / –
Pyruvate	+	+
Sorbitol	+	+
Succinate	+	+
Tartrate	–	–
Valerate	+	+
<i>Electron donor</i>		
Sulfide	–	+ / –
Thiosulfate	+	–

^aSymbols: +, positive in most strains; –, negative in most strains; + / – variable in different strains; nd, not determined.



FIGURE BXII.α.247. Thin-section electron micrograph of *Rhodobium orientis* (strain BM 312) showing internal photosynthetic membranes of lamellar type (indicated by arrow). Bar = 0.5 μm. (Reprinted with permission from A Hiraishi et al., International Journal of Systematic Bacteriology 45: 226–234, 1995, ©International Union of Microbiological Societies.)